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The Effects of Preterm Birth and Intrauterine Growth Restriction on Kidney Development and Renal Function in the Neonate

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DOCTOR OF PHILOSOPHY

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SUMMARY

Preterm birth is defined as birth prior to 37 completed weeks of gestation. In Australia, the incidence is approximately 8% and even higher rates are seen amongst the Australian Indigenous population (approximately 14%). Premature infants are born with organs that are still immature and this is particularly important for kidney development, as the majority of nephrons are formed late in gestation. In addition, a common co-morbidity of preterm birth is intrauterine growth restriction (IUGR); these infants are born small for their gestational age and have poor organ growth *in utero.* This thesis includes a number of studies that aim to gain further understanding of normal kidney development and kidney development accompanied by IUGR during late gestation, as well as the effect of preterm birth and/or IUGR on the development and function of the kidneys after birth.

In chapter two, the temporal and spatial development of nephrons was explored in the developing human kidney. The findings demonstrate a wide variation in the timing of the cessation of nephrogenesis and in the number of glomerular generations formed within the developing human kidneys. Furthermore, once glomeruli were fully formed, the proportion of glomerular cell types (including podocytes, endothelial and non-podocyte cells), remained consistent from mid gestation to term.

When birth comes early, the kidneys compensate for the increased postnatal functional demands by hypertrophy of the kidneys and the glomeruli. However, the mechanisms of how the glomeruli enlarge in the preterm kidney is poorly understood. In chapters three and four, potential ways in which the glomeruli hypertrophy were explored. In chapter 3, the growth of the glomerular capillaries after birth was examined in lambs born moderately preterm and compared to lambs born at term; the effect of mechanical ventilation after birth was also investigated. The findings of this chapter show that being born leads to glomerular capillary

growth, with the length and surface area of the glomerular capillaries at 3 days after birth significantly increased when compared to age-matched gestational controls. After three days of life, there was similar glomerular capillary growth in the term and preterm kidneys that were ventilated for three consecutive days after birth; however, ventilation was shown to negatively impact glomerular capillary growth in the term kidneys. Of concern, preterm birth followed by postnatal ventilation (which is the scenario most common in the clinical setting) led to glomerular capillary dilatation, a marked reduction in the length of the glomerular capillaries and an overall reduced total renal filtration surface area when compared to term non-ventilated lambs.

In chapter 4, the cellular mechanisms leading to the glomerular hypertrophy in the kidneys of preterm infants was further explored, to see whether there were differences in the cellular composition of glomeruli of preterm kidneys relative to kidneys from term-born infants, with particular emphasis on the number of podocytes. Podocytes play a key role in glomerular filtration, and their depletion is linked to glomerular dysfunction and renal disease. A case study of five infants (two term still born infants and three preterm infants that lived for 5-6 weeks after birth) was conducted, whereby the absolute number and proportion of podocytes and non-podocyte cells in the glomeruli of the preterm infants relative to the kidneys from the term-born infants was measured using confocal microscopy and stereological techniques. Interestingly, although there appeared to be glomerular hypertrophy in the kidneys of the preterm infants, this did not appear to be the result of cellular proliferation of either podocyte cells or non-podocyte cells (endothelial, mesangial and inflammatory cells); therefore, suggesting that it is cellular hypertrophy and/or extracellular matrix deposition that leads to the glomerular hypertrophy in the preterm kidneys.

IUGR is a common antecedent and co-morbidity of preterm birth. Chapter 5 examines the effect of IUGR on the development of the human fetal kidney, and on renal function in the neonatal period when it is combined with preterm birth. It was shown that IUGR had an adverse effect on kidney growth, both *in utero* and over the first month of postnatal life. Overall, IUGR led to a significant reduction in the number of glomerular generations formed within the kidney, which likely reflects a reduced nephron endowment. Postnatally, when IUGR was combined with preterm birth, there was evidence of increased tubular injury over the first month of life compared to preterm non-IUGR infants.

Finally, in Chapter 6 markers of renal injury were examined in Indigenous and non-Indigenous preterm infants during the first month of life. Indeed, there is an exceptionally high incidence of renal disease in Indigenous Australians and recent evidence suggests that the antecedents may originate very early in life. Indigenous infants that were born preterm demonstrated a marked increase in the prevalence to renal injury. Abnormally high levels of cystatin - C and neutrophil gelatinase - associated ligase (NGAL), (markers of renal injury) were more prevalent in the Indigenous preterm infants at 4 days of age. Subsequently, Indigenous infants demonstrated increased vulnerability to glomerular injury (represented by abnormally high levels of urinary total protein and albumin) and tubular injury (represented by abnormally high levels of urinary β2-microglobulin) compared to non-Indigenous infants in the first few weeks of life. In addition, the Indigenous infants exhibited a higher incidence of overt renal dysfunction; for example, 13% of Indigenous infants exhibited proteinuria at one of the time points studied during the first month of life compared to 4% of the non-Indigenous preterm infants.

In conclusion, the findings of this thesis have contributed to our understanding of kidney development during late gestation and the effects of preterm birth on renal function in the

neonate. It is shown that IUGR leads to adverse effects on kidney development, and exacerbates renal dysfunction postnatally when combined with preterm birth. Our findings, of increased vulnerability to preterm birth in the kidneys of Indigenous infants, is of concern and warrants further investigation. The findings of this thesis also advance our understanding of the mechanisms leading to glomerular hypertrophy in the early postnatal period in the kidneys of preterm infants. Overall, the findings of this thesis demonstrate the vulnerability of the kidneys to preterm birth, and to its antecedents, and thus highlight the need for future exploration in this clinically important area of research.

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 Chapter 3 of this thesis were previously documented in a Ph.D. thesis by Dr. Megan Sutherland (Preterm birth: Effects on renal development and function, Monash University, 2012). In continuation to the study, I have re-analysed the kidneys, additional animal groups were included and I conducted all of the experimental work (except animal studies) and analysis of data for publication. Dr. Megan Sutherland, contributed equally to the written manuscript relating to this work. To note, Dr. Megan Sutherland is an author to other publications that are to be submitted (Chapters 5 and 6); this work was not a part of her PhD thesis.

With the exception of the material 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 two published review articles, one original paper published in a peer reviewed journal, two papers submitted for publication and two papers in a form ready for submission. It is to be noted that I have used my married surname (Ryan) in all publications and manuscripts. The core theme of the thesis is the effect of preterm birth and intrauterine growth restriction on kidney development and function during the early neonatal period. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of

myself, the candidate, working within the Development and Stem Cells Program of the Monash Biomedicine Discovery Institute and the Department of Anatomy and Developmental Biology, School of Biomedical Sciences, under the supervision of Professor M. Jane Black and Professor Rosemary Horne.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research. In the case of Chapters 2, 3, 4, 5 and 6 my contribution to the work involved the following:

PUBLICATION DECLARATION

Thesis chapter	Publication titles	Publication status	Nature and extent (%) of students contribution
2	Late gestational development of the human fetal kidney: wide variation in the timing of the cessation of nephrogenesis	Submitted	Conducted the majority of the experimental analyses, performed all data analyses, and wrote the manuscript. Total contribution: 80%
3	Effects of preterm birth and ventilation on glomerular capillary growth in the neonatal lamb kidney	Published	Performed all of the experimental work (except for animal studies), performed all data analyses, and co-first authored the manuscript. Total contribution: 50%
4	A case study comparing the cellular composition of	Submitted	Performed all of the experimental work,

	glomeruli in infants born term and preterm		performed all data analyses, and wrote the manuscript. Total contribution: 80%
5	Intrauterine growth restriction: impact on nephrogenesis and on renal function in the preterm neonate	Manuscript Drafted	Performed the majority of the experimental work, performed all data analyses, and wrote the manuscript. Total contribution: 80%
6	Renal impairment already evident within the first month of life in preterm Indigenous Australians	Manuscript Drafted	Performed all of the experimental work, performed all data analyses, and wrote the manuscript. Total contribution: 80%

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

Student signature: [redacted] Date: 21.9.16

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

Main Supervisor signature: [redacted] Date: 21.9.16

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ABBREVIATIONS

AGA - Appropriate for gestational age

AIHW - Australian Institute of Health and Welfare

AKI - Acute kidney injury

ANCOVA - Analysis of co-variance

ANOVA - Analysis of variance

ATPase - Adenylprophosphatase

BP - Blood pressure

BPD - Bronchopulmonary dysplasia

BSA - Body surface area

C - Cloaca

C_{Cr} - Creatinine clearance

CKD - Chronic kidney disease

CLD - Chronic lung disease

CO_2 - Carbon dioxide

CPAP – Continuous positive airway pressure

Cr - Creatinine

ESKD - End stage kidney disease

ESRD - End stage renal disease

ELISA - Enzyme-linked immunosorbent assay

FE_{Na} - Fractional excretion of sodium

FIRS - Fetal inflammatory response syndrome

Flt -1 - fms-like tyrosine kinase 1

FSGS - Focal segmental glomerulosclerosis

g - Grams

GA - Gestational age

GBM - Glomerular basement membrane

GDNF - Glial cell line-derived neurotrophic factor

GFR - Glomerular filtration rate

H^+ - Hydrogen ion

IL-18 - Interleukin-18

IM - Intermediate mesoderm

IUGR - Intrauterine growth restriction

HIF - Hypoxia - inducible factor

HMW - High molecular weight

K^+ - Potassium ion

KIM - Kidney injury molecule 1

LMW - Low molecular weight

mg - milligram

mm - millimeter

Na^+ - Sodium ion

ND - Nephric duct

NEC - Necrotising enterocolitis

NHMRC - National Health and Medical Research Council of Australia

NICU - Neonatal intensive care unit

NGAL - Neutrophil gelatinase associated lipocalin

NPCs - Non-podocyte cells

NSAID - Non-steroidal anti-inflammatory drug

O_2 - Oxygen

PA - Postnatal age

PDA - Patent ductus arteriosus

PPROM - Premature pre-labour rupture of membranes

RC - Renal corpuscle

RDS - Respiratory distress syndrome

SBP – Systolic blood pressure

SD - Standard deviation

SEM - Standard error of the mean

SGA - Small for gestational age

SpO2 - Blood oxygen saturation

UTP - Urine total protein

VEGF - Vascular endothelial growth factor

VEGFR - Vascular endothelial growth factor receptor

vWF - Von Willebrand factor

WD - Wolffian duct

WHO - World Health Organisation

WT-1 - Wilm's tumor suppressor gene-1

β2-M - βeta-2-microglobulin

µ Micron

% Percentage

Chapter One:
LITERATURE REVIEW

1. PRETERM BIRTH

Preterm birth occurs in approximately 11% of all births and is one of the leading causes of neonatal morbidity and mortality worldwide (Blencowe et al., 2012). Preterm birth is defined as birth prior to 37 completed weeks of gestation, with birth from 37 completed weeks to 42 weeks of gestation considered as full term (Tucker and McGuire, 2004). Preterm birth can be further sub-classified into moderately preterm, very preterm and extremely preterm. Moderately preterm infants are classified as those born from 32 up to 37 weeks of gestation, very preterm births are those born from 28 up to 32 weeks' gestation, extremely preterm births are those born before 28 weeks' gestation (Refer to Figure 1) (Goldenberg et al., 2008). Babies born prior to 23 weeks usually do not survive. The majority (60-70%) of preterm newborns are born from 34 to 36 weeks of gestation. The incidence of preterm infants born at 32-33 weeks gestation is ~20% and ~15% are born at 28-31 weeks, preterm birth prior to 28 weeks is the least common (Goldenberg et al., 2008).

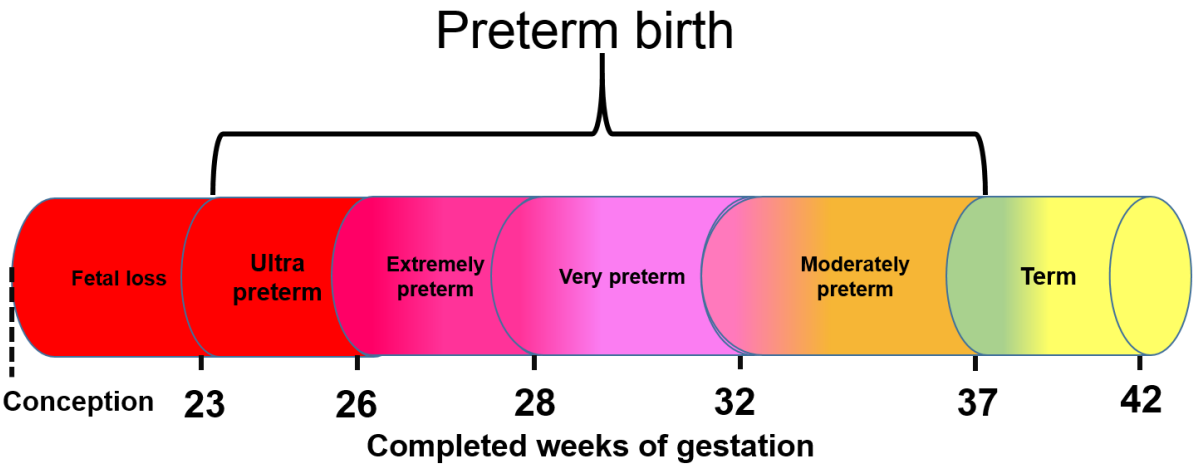


Figure 1: Sub-classifications of preterm birth. Preterm birth is defined as birth prior to 37 completed weeks of gestation. Preterm birth can be further sub classified into moderately preterm, very preterm, extremely preterm and ultra preterm. Term infants are born after 37 completed weeks to 42 weeks of gestation.

1.1 INCIDENCE OF PRETERM BIRTH

The global number of preterm deliveries each year has been slowly increasing and at the present time it is around 11% of births worldwide (Blencowe et al., 2012). In the USA the incidence of preterm birth is ~12.3% (Mathews et al., 2011), in Europe it is 5-7% (Beck et al., 2010), and in Australia it is ~8.2% (Laws et al., 2010). However, within these populations some ethnic groups have a higher incidence of preterm birth. For example in African Americans the incidence of preterm birth is high at ~17.5% (Martin et al., 2011) and in Indigenous Australians 13.3% of all births are preterm (Laws et al., 2010). Of concern, the prevalence of preterm birth in developing countries is very high; for example, up to ~17.5% of the reported births in South Africa are preterm and this is likely to be even higher as many births are not recorded (Beck et al., 2010).

Survival following preterm birth (especially in those born very and extremely preterm) has improved dramatically since the first introduction of neonatal intensive care units (in the 1960s). With subsequent refinements in prenatal and neonatal care, newborns born as early as 25 weeks' gestation now have an 80% chance of survival (Allen et al., 1993, Kutz et al., 2009). In particular, the use of antenatal/neonatal corticosteroids (which accelerate lung maturation in the newborn) and surfactant therapy (which reduces alveolar surface tension in the presence of respiratory distress syndrome) have facilitated the recent improvement in survival (Roberts and Dalziel, 2006).

1.2 CAUSES OF PRETERM BIRTH

The cause of premature delivery is multifactorial and differs with each pregnancy. It can occur spontaneously or be the result of emergency induced delivery. The most common identified causes of spontaneous preterm delivery are onset of premature labour (45%), and premature

pre-labour rupture of the membranes (25%) (Goldenberg et al., 2008). The main identified cause of emergency induced delivery is maternal and fetal infection (35%) (Goldenberg et al., 2008). To date, the etiological mechanisms leading to spontaneous preterm labour and premature pre-labour rupture of the membranes are not well defined. There are a number of risk factors associated with increased risk of preterm delivery (Goldenberg et al., 2008). Pregnancy complications that often lead to emergency induced preterm delivery include: Chorioamnionitis, placental insufficiency/abruption (Barros et al., 2015), pre-eclampsia (Ananth et al., 1997, Thornton, 2013), oligohydramnios (abnormal amniotic fluid levels) (Coolen et al., 2010, Hernandez et al., 2012) and intrauterine growth restriction (IUGR); IUGR is often a co-morbidity of these other pregnancy complications. In addition, other factors associated with maternal health that can also lead to an increase in the risk to premature delivery include: multiple pregnancies (Lynch et al., 2003, Chang et al., 2011a), previous preterm birth (Blondel et al., 2002, Smith, 2003, Buchmayer et al., 2004, Goldenberg and Culhane, 2006, Mercer et al., 2006, Brown et al., 2008); assisted fertility (Barlow et al., 1988, Hill et al., 1990, Ventura et al., 1998, Blondel et al., 2002); low/high BMI (Scholl et al., 1988, Hendler et al., 2005, Torloni et al., 2009, Aly et al., 2010, Madan et al., 2010); depression (Peacock et al., 1995, Alder et al., 2007, Gavin et al., 2009, Poehlmann et al., 2009); cervical incompetence (Mancuso et al., 2011, Manuck et al., 2011); asthma (Powell et al., 2013); diabetes (Ray et al., 2001, Vangen et al., 2003, Rosenberg et al., 2005) and hypertension (Seely and Maxwell, 2007).

Collectively, the management of women that are of “high risk” needs to be appropriately addressed to improve the approach towards creating the best outcome for these women and their babies, from the time of conception to the time of delivery.

Although there has been a marked improvement in the survival of preterm infants over recent decades, preterm birth still remains the leading cause of infant mortality and morbidity. Perinatal mortality is currently around 6 to 8.5 times higher in preterm infants than in term infants (Pulver et al., 2009). Preterm infants are vulnerable to many postnatal complications due to the increased functional demands in the extra-uterine environment, at a time when the immature organs are ill-equipped for the transition to life ex-utero.

1.3 POSTNATAL CLINICAL CARE OF PRETERM INFANTS

1.3.1 HYPEROXIA AND VENTILATION

In utero, the fetus normally develops in a relatively hypoxic environment (5% oxygen) and this facilitates vascular and cellular development in organs such as the kidney (Rodesch et al., 1992, Fischer and Bavister, 1993, Tufro-McReddie et al., 1997). At birth, the neonate is exposed to an abrupt increase in oxygen from ~5% to 21% (Tufro-McReddie et al., 1997). The blood oxygen saturation levels (SpO_2) rise from 45-55% in the fetus (Finer et al., 2010) to 80-90% in the first five minutes after birth (Kamlin et al., 2006). Hence, when a baby is born preterm, organ development no longer ongoing in a hypoxic environment and therefore, it is likely that this will lead to deleterious effects on growth.

In addition, in the preterm neonate the lungs are very immature at the time of birth; therefore, the neonate requires resuscitation and ongoing ventilation (AHA and AAP, 2006). Exposure to supplemental oxygen therapies, can lead to exposure to very high concentrations of O_2 (up to 100%), in an attempt to normalise blood oxygen levels (Tan et al., 2005, Rabi et al., 2011). However, during this process the infant can experience high blood oxygen concentrations (often only transitory) until the blood oxygen levels become normalised. Of concern, hyperoxia can lead to oxidative stress of the neonate, which has been shown to subsequently cause

cellular injury and cell death in response to accumulation of free radicals and thereby exhaustion of antioxidants (Vento et al., 2003, Winterbourn, 2008). Consequently, this can lead to a number of common morbidities of prematurity such as retinopathy of prematurity, necrotizing enterocolitis and bronchopulmonary dysplasia (Saugstad, 2001). In the kidney of the human neonate, oxidative stress has been reported to cause renal tubular injury (Perrone et al., 2007) and it has been linked to impairment of nephrogenesis (development of nephrons in the kidneys) in animal studies (Yzydorczyk et al., 2008). In the rat model (where nephrogenesis is ongoing in the first two weeks after birth), a significant reduction of nephrons (25%) was reported in adulthood (25-35 weeks of age) (Yzydorczyk et al., 2008) following exposure to 80% oxygen during the early postnatal period.

1.4 EFFECT OF PRETERM BIRTH ON IMMATURE ORGANS

The increasing awareness of the potential adverse effects of being born early on immature organ systems has led to many studies over recent years looking at the consequences of preterm birth on fetal organ development, such as in the lungs (Kallapur and Ikegami, 2006), brain (Goldenberg et al., 2000, De Felice et al., 2001), cardiovascular system (Bensley et al., 2010, Bensley et al., 2012, Abdel-Hady et al., 2013); and the kidneys (Rodriguez et al., 2004, Gubhaju et al., 2009, Gubhaju et al., 2011, Sutherland et al., 2011).

1.4.1 LUNG INJURY

Preterm infants are born at a time when lung development is still ongoing. This causes preterm newborns to be born with immature lungs that are abruptly exposed to increased oxygen saturation concentrations in the extra-uterine environment. For this reason, preterm infants born < 32 weeks' gestation, are forced to breathe air before alveolarisation of the lung has begun, thus leading to very insufficient gas exchange and lung injury. As a consequence, these

preterm infants may develop neonatal respiratory distress syndrome (RDS), due to deficiency in pulmonary surfactant, which is important to maintain alveolar surface tension (Fraser et al., 2004). Subsequently, these infants require mechanical ventilation, which has been shown to increase the risk of developing bronchopulmonary dysplasia (BPD) (Northway et al., 1967) and severe lung injury (Van Marter et al., 2002), as well as leading to possible long-term respiratory problems, including chronic lung disease (CLD) and asthma (Moss, 2006, Mwansa-Kambafwile et al., 2010, Been et al., 2014). In a study by Picone and Paolillo, (2010), which evaluated respiratory problems, (specifically, respiratory distress syndrome, transient tachypnea, pneumonia, pneumothorax and apnea), out of 417 late-preterm infants over a period of two and a half years, it was found that 24% of infants experienced asphyxia, which required ventilation; 20% presented with a respiratory illness in the first day of life; 10.8% developed RDS and 0.5% developed pneumonia. Out of the infants born at 34 weeks' gestation, all of them required ventilator support and 13.3% of these developed a higher incidence of respiratory distress syndrome (Picone and Paolillo, 2010). Furthermore, extremely preterm infants may require additional surfactant (Smith et al., 2010), as well as corticosteroids (Mwansa-Kambafwile et al., 2010, Roberts et al., 2013) to facilitate the development of the immature lungs.

1.4.2 NEUROLOGICAL IMPAIRMENT

The degree of neurological impairment after preterm birth highly depends on the gestational age and weight of the infant at birth. Consequently, the earlier the gestational age and the lower the birth weight, the greater the risk for developing neurological problems such as cerebral palsy (Goldenberg and Culhane, 2007). The prevalence of cerebral palsy in infants born at 24- 30 weeks gestation is about 100 per 1000 live births (Vincer et al., 2006).

Alternatively, in term infants of 2500g at birth the prevalence is reduced to about 1-2 per 1000 births (Goldenberg and Culhane, 2007). In a cohort of 576 babies born extremely preterm (from 22 to 25 weeks' gestation) that were evaluated after birth in 2006, 13.4% were categorised as having severe neurological impairment and 11.8% had moderate impairment (Moore et al., 2012). Overall, 38% of the children had some degree of neurodevelopmental impairment, including 23% with motor impairment and 24% with developmental impairment. The proportion of mental disability for each age was 45% in babies born at 22-23 weeks gestation, 30% at 24 weeks gestation, 25% at 25 weeks and 20% at 26 weeks gestation. Similar findings in other long-term studies have shown 21-29% of children born prematurely (from 23 to 27 weeks gestation) are diagnosed with moderate to severe cerebral palsy (Mikkola et al., 2005, Farooqi et al., 2006). Correspondingly, a systematic review and meta-analysis on the overall prevalence of cerebral palsy reported that the cerebral palsy was highest in children before 28 weeks gestation (Oskoui et al., 2013). Other common brain injuries prevalent in preterm infants include intraventricular haemorrhage, which results from a decrease in cerebral blood flow, and periventricular leukomalacia. These injuries are said to occur in 20-25% of premature infants weighing less than 1500g (Volpe, 2008).

1.4.3 CARDIOVASCULAR INJURY

Preterm birth occurs at a time when cardiomyocytes (functional units of the heart) are still maturing. For this reason, preterm infants are born when their cardiovascular system is still immature and is ill adapted to the abrupt changes associated with the haemodynamic transition that occurs in the immediate period after preterm birth, including a sudden increase in blood pressure. Consequently, this immaturity of the blood vessels can lead to cardiovascular complications (such as patent ductus arteriosus) (Stoll et al., 2010, Abdel-Hady

et al., 2013). Patent ductus arteriosus results from the non-closure of the shunt that connects the pulmonary trunk to the aorta in the fetus. During normal development *in utero*, the oxygenated blood bypasses the pulmonary circulation through the ductus arteriosus. After 48 hours of postnatal life, the shunt closes due to the increase in systemic arterial pressure (Schneider and Moore, 2006), where a rapid increase in pulmonary blood flow occurs, from 138 ml/min/kg in the fetus to 245 ml/min/kg in the newborn (Gao and Raj, 2010). This increase in blood flow causes the returning blood from the pulmonary vein to increase the pressure in the left ventricle of the heart, causing the left ventricle to become the dominant ventricle after birth (Blackburn, 2007). However, it is quite common for preterm infants to experience a delay in the closure of the shunts, which can cause the preterm infant to become hypotensive (possess low arterial pressure) immediately after birth (Dannevig et al., 2005). This may result in insufficient circulation to organ systems, poor cardiovascular function and heart failure (Dice and Bhatia, 2007). In extremely immature infants, there is increased evidence of further delay to the closure of the ductus arteriosus, subsequently causing a delayed rise of the systemic arterial pressure, resulting in reduced cardiac output and overall poor blood flow between the heart and lungs (Abdel-Hady et al., 2013). Patent ductus arteriosus has also been associated with increasing the risk of bronchopulmonary dysplasia and intraventricular haemorrhage in the brain (Ohlsson and Shah, 2011). In addition, immaturity of the major blood vessels as a result of preterm birth has been linked to long-term predisposition of cardiovascular disease and hypertension (Burke et al., 1995, Davis et al., 2001, Johansson et al., 2005, Polak et al., 2011, Bensley et al., 2012). Recent experimental studies by (Bensley et al., 2010), in an ovine model have shown preterm birth to lead to a significant increase in collagen deposition and altered cardiomyocyte maturation at 9 weeks' postnatal age. Also observed was an increase in

elastin deposition in the walls of the aorta and pulmonary artery following preterm birth (Bensley et al., 2012). Overall, numerous studies have reported an increased risk of the development of hypertension with increased prematurity (Bonamy et al., 2005, Johansson et al., 2005, Doyle, 2008, Norman, 2010, Crump et al., 2011, Kerkhof et al., 2012).

KIDNEY INJURY

There have been a limited number of studies looking at the effect of preterm birth on kidney development in humans (Hinchliffe et al., 1991, Rodriguez et al., 2004, Faa et al., 2010, Sutherland et al., 2011). In the human, preterm birth caused a higher percentage of structurally abnormal glomeruli compared to age-matched fetal stillborn gestational controls (Sutherland et al., 2011). Importantly, preterm birth has shown to cause marked growth in kidney size during the early postnatal period, compared to term infants, and this was accompanied by an increase in glomerular size. However, in order to have greater understanding on the effects of premature birth, intrauterine growth restriction (IUGR), and preterm birth accompanied by IUGR on the developing kidneys during late gestation and over the first month of life; it is important to have a general understanding of normal renal development and function in the human.

2. NORMAL RENAL ANATOMY

2.1 KIDNEY DEVELOPMENT

2.1.1 THE NEPHRON

The basic functional unit of the kidney is the nephron. The nephron is responsible for regulating water content and electrolytes, thus maintaining blood osmolality and blood volume, excreting waste products and regulating blood pressure (Michos, 2009). The nephrons are formed in early development of the kidney with no new nephrons formed after birth. Kidney

development initially involves the formation of two transitory and primitive kidney structures, the pronephros and mesonephros, followed by the formation of the metanephros, which becomes the permanent kidney (Figure 2) (Lumbers, 1995).

2.1.2. PRONEPHROS AND MESONEPHROS

The pronephros is a vestigial structure seen in early fetal life, approximately day 20-22 in the human embryo (Oliver, 1968)(Figure 2A). It regresses in approximately the fourth week of gestation (Ludwig and Landmann, 2005), as the mesonephros forms and becomes responsible for renal function during early fetal development (Michos, 2009), producing small volumes of urine at 6 to 10 weeks' gestation (Woolf et al., 2003). During the formation of the mesonephros, a pivotal component of the developing excretory system is formed, termed the nephric (Wolffian) duct (ND; Wolffian duct (WD)) (Figure 2B). Briefly, during embryogenesis, the intermediate mesoderm (IM), which consists of metanephric mesenchyme, gives rise to the nephric epithelium and collecting system; where firstly, specification occurs in the IM, followed by caudal extension, formation of a simple epithelial tube, and the conversion of the caudal segmental of the tube to a pseudostratified epithelium (Costantini and Kopan, 2010). More specifically, the cells in the dorsal IM coalesce into nephric duct and from the ventral IM, the mesechanymal cells remain undifferentiated forming the nephric cord (Costantini and Kopan, 2010). The interaction between the ND and adjacent nephric cord are imperative for the first transient group of primitive tubules to form. The mesonephric tubules form rostro-caudally along the duct, extending medially from the duct into the nephric cord, whilst the Wolffian duct elongates dorsally towards the cloaca (Figure 2C) (Dressler, 2006). Subsequently, the nephric duct gives rise to the first ureteric bud (UB) tips following inductive Wnt signalling from the metanephric mesenchyme (Carroll et al., 2005)(Figure 2D). Another important

signaling pathway involved in the cell movements in the ND that lead to the formation of UB is the interaction between the Ret receptor on the ureteric bud, and its mesenchymal ligand glial cell line-derived neurotrophic factor (GDNF) (Chi et al., 2009). It is well established in the literature that GDNF/Ret signaling plays a crucial role in ureteric budding, where changes in the expression of GDNF can have detrimental effects (reviewed in Constantini (2010). Additionally, a gene regulator which acts as an inducer during this process is the SOX gene, which is essential for maintaining correct outgrowth patterns during kidney development (Reginensi et al., 2011).

2.1.3 METANEPHROS

The metanephros (permanent kidney; Figure 2C) begins its development at approximately 20-27 days gestation, before the two transient stages have regressed completely (Lumbers, 1995). At this point, the epithelial cells at the caudal end of the Wolffian duct elongate towards the metanephric mesenchyme, where a dense mass of embryonic cells are found at the base of the nephrogenic cord, forming the ureteric bud (Yosypiv, 2008). Reciprocal interactions occur between the ureteric bud and metanephric mesenchyme (Figure 2D), that are important for nephron formation and tubule growth (al-Awqati and Goldberg, 1998). The mesonephros degenerates by 35 to 84 days gestation, and remaining mesonephric structures are involved in genitourinary development (Sweeney and Avner, 2004).

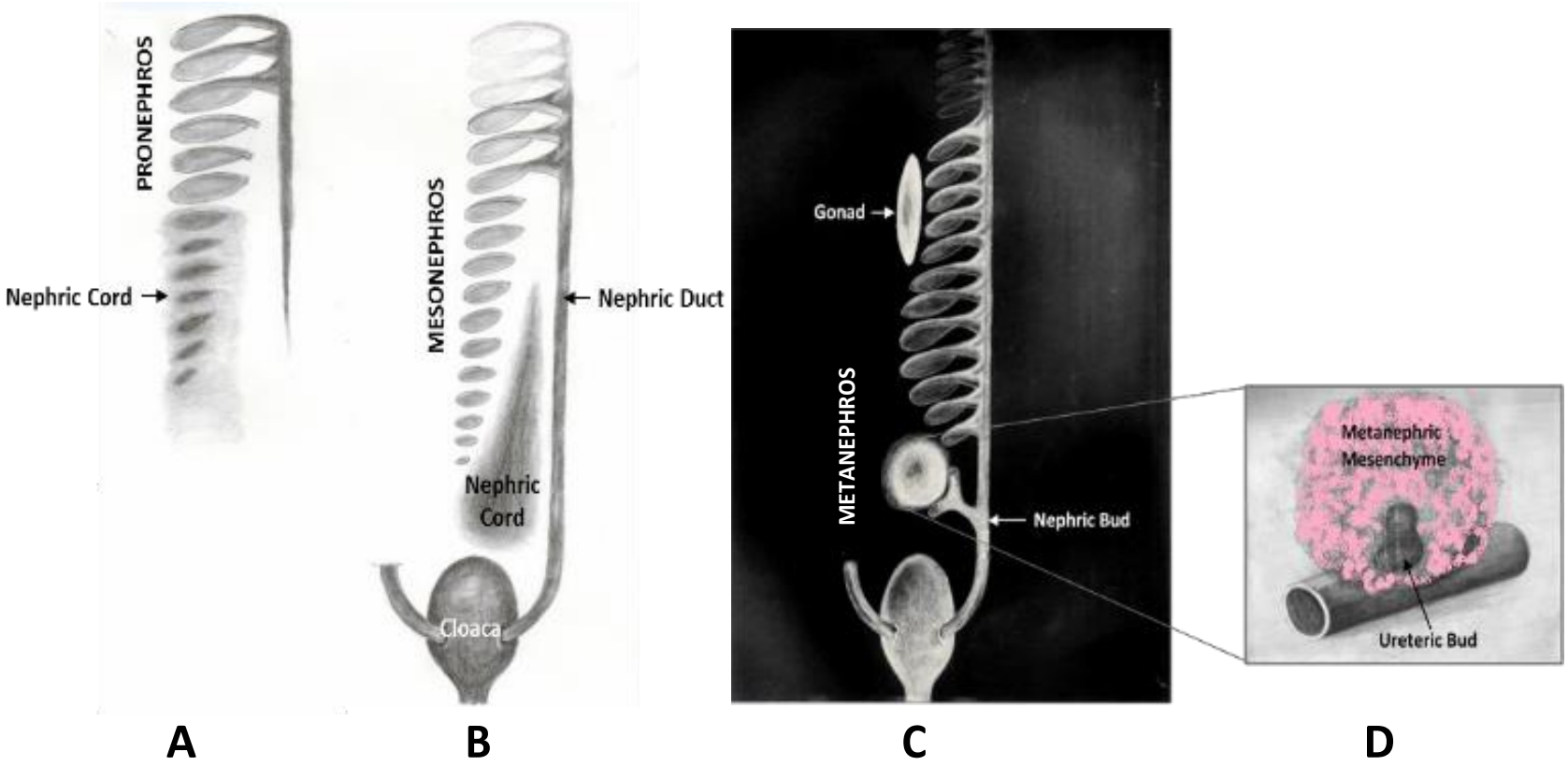


Figure 2. Development of the vertebrae kidney. Three stages of kidney developmental: pronephros (A), mesonephros (B), metanephros (C), and the ureteric bud surrounded by metanephric mesenchyme (D). Adapted from *Developmental Biology, Eighth Edition (Gilbert, 2006).*

2.1.4 BRANCHING MORPHOGENESIS IN THE KIDNEY

Branching morphogenesis of the ureteric tree is the process which ultimately determines the final structure of the adult kidney. The ureteric bud ultimately gives rise to the collecting duct system, including; the ureter, renal pelvis, calyces and collecting tubules, and the metanephric mesenchyme differentiates into the glomeruli, and tubule segments of the nephron and the interstitium (Cullen-McEwen et al., 2016) (Figure 3). Branching morphogenesis occurs as the newly formed ureteric bud tip (ampullae), continues to elongate into the developing mesenchyme. At this point the ampullae begin to divide into two lateral branches (Figure 3A). The first branching is said to be symmetrical, with an angle as wide as 180 degrees. Thereafter, each of the branches continue to bifurcate, either symmetrically or asymmetrically, with the angles gradually reducing with each branching division (Saxen and Sariola, 1987, Osathanondh and Potter, 1963a). It is important to note, that only one half of the newly divided branches continue to further divide, whilst the other half of the branch tip induces nephron formation (Costantini, 2006). The newly formed branches form the collecting tubules, which eventually lead to the formation of the collecting duct (Osathanondh and Potter, 1963b).

After 3-4 generations of ureteric branching (Figure 3B), each induced nephron is attached to a single ampulla of a collecting tubule. As the ampullae continue to divide and form additional collecting tubules, the attached nephrons move simultaneously with the ampullae over subsequent generations. During this time, the ampullae can only produce a nephron if there is no nephron previously attached. This phase of extensive branching reaches 15 generations (al-Awqati and Goldberg, 1998), after which there is no more division. From this time (~15 weeks' gestation), the ampullae stop dividing and can then form new nephrons whilst already having an attached nephron. These newly formed nephrons remain either connected to a single

collecting duct, or to the connecting tubule of another nephron, forming in an 'arcade' manner (Figure 3C). This type of arrangement allows for nephrons to be attached to one another, so that only the recently formed nephrons are attached to the ampullae. Four to seven nephrons per ureteric bud tip are formed in this way (Osathanondh and Potter, 1963b). The terminal portion of each collecting tube is then formed from 20 to 36 weeks' gestation, this is the time when nephrogenesis occurs rapidly and is known to be a critical period in nephrogenesis (Hinchliffe et al., 1991). In this regard, Hinchliffe et al. (1991) demonstrated that about 60% of nephrons are formed during the third trimester of pregnancy. Ultimately, at 32-36 weeks' gestation, the ampullae disappear and nephrons induced by each collecting tubule will form the termination of the tubule. These nephrons are referred to as terminal nephrons, found individually attached to the end of the ureteric branches (Figure 3C). As a consequence of this specific branching process, the most mature nephrons are located near the medulla and the less mature are found in the outer renal cortex (Lumbers, 1995). To date, the spatial regulation of nephrogenesis in the kidney is not well understood. This is an important area of further study with the aim of optimising the number of nephrons formed, and hence the adaptation of the kidneys to the postnatal environment.

2.2 NEPHROGENESIS DURING NORMAL GESTATIONAL DEVELOPMENT

Following initial branching, nephron formation involves reciprocal interactions between the metanephric mesenchyme and the ureteric bud epithelium. These interactions require continuous molecular signaling between the ampullae and the metanephric mesenchyme. At each ampulla, the ureteric buds expand and undifferentiated mesenchymal cells form a metanephric cap. The metanephric cap surrounds the ends of the newly formed buds which eventually form the nephrons. Signals from the ampullae induce a mesenchymal to epithelial

differentiation, forming polarised renal vesicles, which have been recognised as precursors of the nephrons (Michos, 2009) (Figure 3D). The renal vesicles are considered the first epithelial structure originating from the cap mesenchyme. Formation of the mature nephron requires segmentation and patterning of the renal vesicle, followed by the fusion with the ureteric component of the fetal kidney. This process which allows uninduced mesenchyme to condense in response to inductive signals from the ureteric bud is controlled by a gene known as Wilm's tumor suppressor gene-1 (WT-1) (Kreidberg et al., 1993). Another important gene involved in the differentiation of the cap mesenchyme cells into the renal vesicle is Wnt-4. Studies that have used Wnt-4 mutants (Kobayashi et al., 2005), have found that in the absence of Wnt-4 the epithelium of the renal vesicle does not generate.

Nephrogenesis proceeds, as the newly polarised renal vesicles proliferate and produce comma shaped bodies. These bodies differentiate into S-shaped bodies, which in turn, differentiate into tubular structures, the epithelial layers of the Bowman's capsule and the glomerular capillaries (Figure 3D). Once the tubular structures extrude from the glomerular cavity, this allows for proliferation of the glomerular capillaries into the epithelial wall of the Bowman's capsule, forming the Bowman's capsule and glomerular tuft. The structure consisting of the Bowman's capsule, Bowman's space and glomerular capillary tuft is known as the renal corpuscle. Eventually, the renal corpuscle fuses with the collecting duct forming the mature nephron. After 32- 36 weeks gestation, once nephrogenesis has ceased, the developing kidney undergoes further growth and maturation including, glomerulogenesis and vascular development (Little, 2006). Ultimately, the maturation of the components of the nephron continues until 2-3 weeks after birth, where the cortical size increases along with growth of the

loops of Henle (from the last formed glomeruli in the outer renal cortex), thus increasing the size of the medulla (Cullen-McEwen et al., 2016).

2.2.1 GLOMERULOGENESIS

Glomerulogenesis involves the development of the renal corpuscle which arises from the lower limb of the S-shaped body, and this critical process occurs simultaneously with nephrogenesis. The S-shaped body is organised into three segments, the proximal, medial and distal: the proximal cells have been shown to differentiate to form the parietal epithelium of the glomerulus, lining the Bowman capsule and the visceral epithelium of the glomerulus (known as the podocytes) (Osathanondh and Potter, 1963b, Saxen and Sariola, 1987, Dressler, 2006) (Figure 4). Podocyte cells evolve from columnar epithelial cells from the S-shape body, and are a crucial component of the glomerular filtration barrier which consists of three layers: capillary endothelium, glomerular basement membrane, and podocytes (Figure 4). Endothelial precursor cells come from the mesenchyme and differentiate to form the capillary loop within the lower cleft of the S-shaped body (Abrahamson and Wang, 2003). The podocyte epithelium and the developing endothelial cells are both involved in producing the components required to form the glomerular basement membrane, and continue to maintain its structure during glomerular maturation (Abrahamson and Robert, 2003, Eremina and Quaggin, 2004). The glomerular basement membrane is a specialised extracellular matrix that is a crucial component of the filtration barrier, and it separates the vasculature from the filtrate. Studies on lamini alpha5 mutant and transgenic mice (Miner et al., 1997) have alluded to the importance of the glomerular basement membrane in glomerulogenesis.

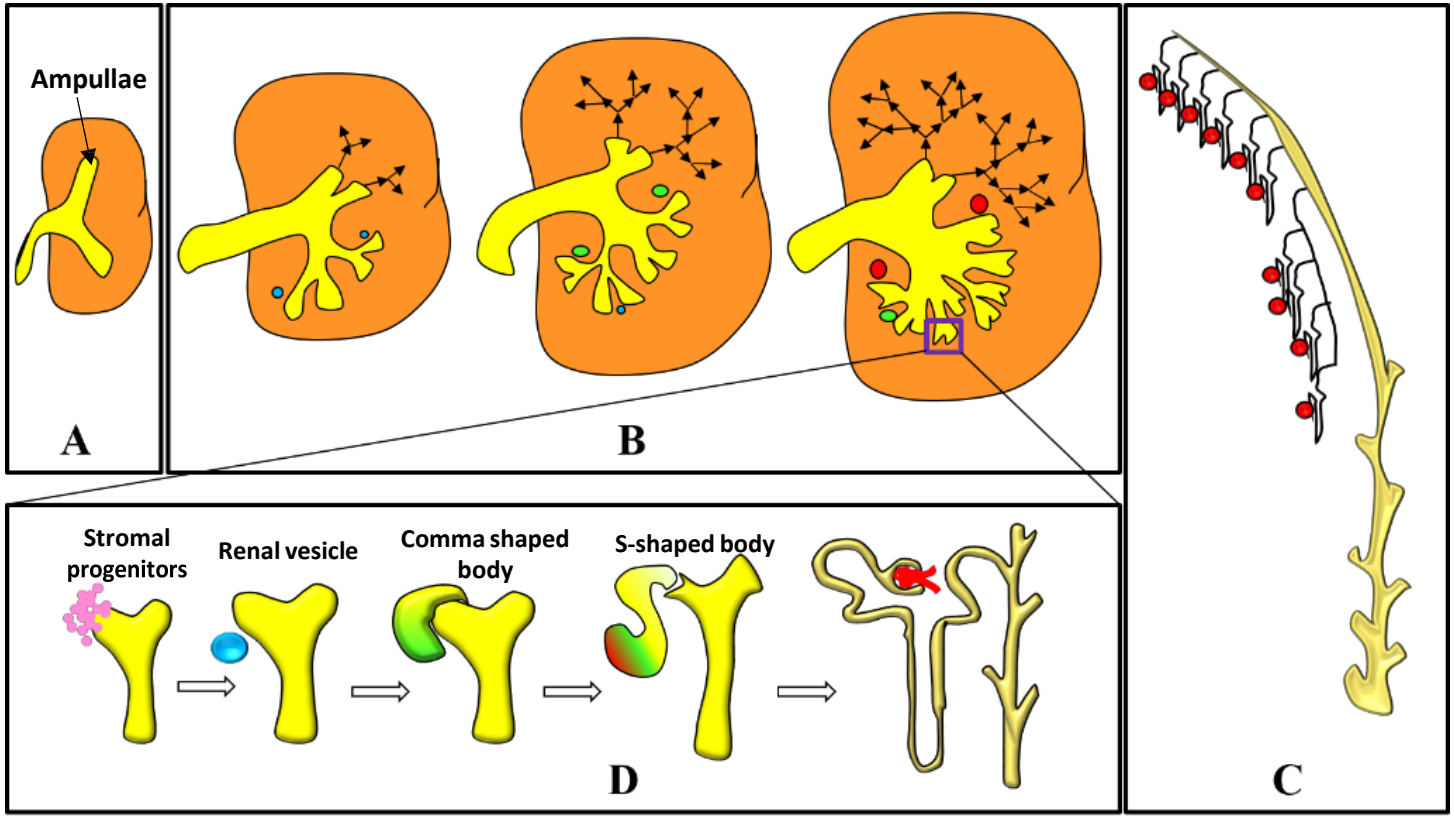


Figure 3.Nephrogenesis. Schematic of the stages of branching morphogenesis and nephrogenesis in the developing kidney, based on Osathanondh and Potter (1963b). Initially the ureteric bud ampullae begin to divide into two lateral branches (A). Thereafter, the branches continue to bifurcate up to 3- 4 generations of ureteric budding (B). Simultaneously, nephrons are induced at the branch tips (ampulla), where only one half of the branch tip induces nephron formation (B). These newly formed nephrons remain either connected to a single collecting duct, or to the connecting tubules of another nephron, forming in an 'arcade' manner (C). Signals from the ampullae induce a mesenchymal to epithelial differentiation, forming the renal vesicles, which in turn, produce the comma-shaped and S-shaped bodies, and finally differentiate and form the tubular structures, Bowman's capsule and glomerular capillaries (D). Vesicles are shown in blue, comma/ S-shaped bodies are shown in green and mature nephrons are red.

These studies have shown that in the absence of the glomerular basement membrane the three glomerular cell types become unstable within the glomerulus, leading to glomerular disorganisation and failed glomerular vascularisation (Miner et al., 1997). The podocytes attach their foot processes to the glomerular basement membrane linking juxtaposed foot processes to one another, allowing for glomerular maturation (Abrahamson and Robert, 2003, Eremina and Quaggin, 2004). The glomerular endothelial cells are responsible for lining the glomerular capillaries. These endothelial cells bear many fenestrations which are essential to allow for fluid to cross the cell layer (Miner, 2011). During *in situ* vasculogenesis, endothelial cells differentiate from mesenchymal or epithelial cells within the lower part of the S-shaped body (Robert et al., 1996). Glomerular capillary development initiates with a single capillary loop that sprouts into the glomerular cleft, found between the primitive podocytes and proximal tubule of the S-shaped body (Potter, 1965). The capillary loop then divides into 6-8 loops, in conjunction with glomerular maturation (Potter, 1965). The medial segment of the S-shaped body forms the proximal tubules, whilst the distal portion is responsible for the fusion process with the collecting tubules (Georgas et al., 2009).

The mesangial cells are found adjacent to endothelial cells on the glomerular basement membrane. These cells originate from cap mesenchymal cells which are responsible for the formation of the non-nephron lineage and are suggested to constitute approximately 30-40% of the glomerular cell population (Kobayashi et al., 2008). The adhesion between the mesangial cells to the glomerular basement membrane is crucial in the process of capillary looping, which is the major characteristic of the glomerular tuft (Kikkawa et al., 2003). Furthermore, mesangial cells are involved in assisting the glomerular vasculature to respond to various physical stimuli (Schlondorff, 1987). Once mature, the glomerular capillary tuft consists of several capillary

loops with mesangial cells found both at their base as well as into each capillary branch (Potter, 1965). The precise mechanisms that differentiate cell types along the lineage, such as differentiation into mesangial cells, however, remains largely unknown (Dressler, 2009).

2.2.2 VASCULAR ENDOTHELIAL GROWTH FACTOR AND GLOMERULOGENESIS

Vascular endothelial growth factor (VEGF) A is an important regulator of blood vessel growth and is highly involved in the development and functioning of the glomerulus. VEGF-A, B, C, D and the placental growth factor all belong to the same family of growth factors (Eriksson and Alitalo, 1999).VEGF-A is crucial in the establishment of the glomerular filtration barrier. This is shown in its ability to promote endothelial cell migration, proliferation and survival (Eremina and Quaggin, 2004). VEGF-A is also involved in the formation and maintenance of podocyte cells during podocyte differentiation (Eremina and Quaggin, 2004) and plays a pivotal role in the regulation of angiogenesis as it has been shown to regulate vascular permeability, endothelial cell migration proliferation and survival (Eremina and Quaggin, 2004).

In the developing human, VEGF expression is first detected during the S-shaped body stage of glomerulogenesis, within the podocyte precursor cells (Simon et al., 1995). During this stage the podocyte precursor cells are columnar-shaped cells, located adjacent to the vascular cleft. During glomerular filtration barrier formation, cells that express the vascular endothelial growth factor receptor VEGFR-1 (also known as Flt-1; fms-like tyrosine kinase 1), at this time point are found in the renal mesenchyme and they migrate into the vascular cleft, proliferate and differentiate alongside VEGF-positive cells (Abrahamson, 1991), thus stimulating blood vessel growth.

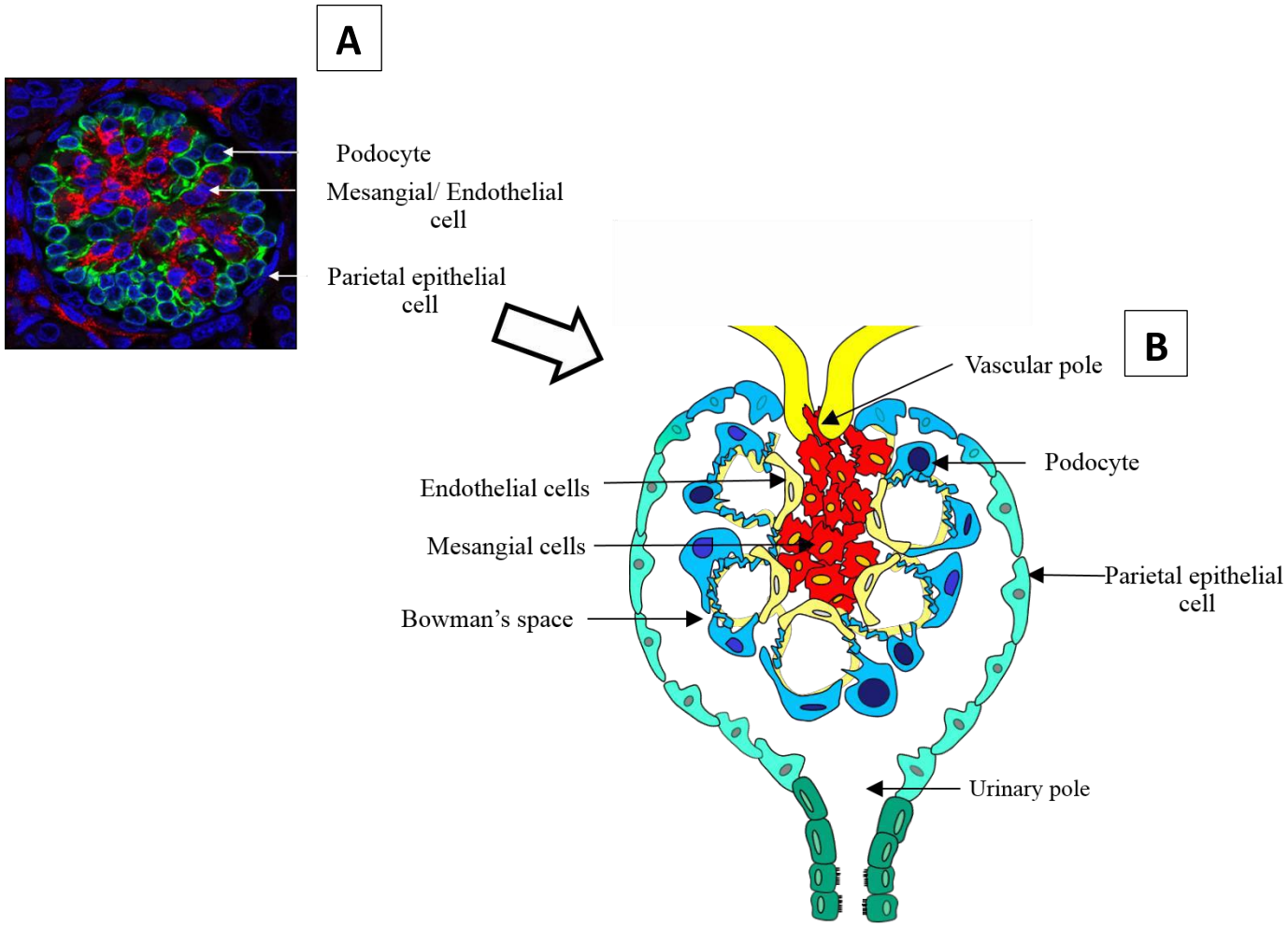


Figure 4. Glomerulus. Immunolabelled glomerulus (A); cell nuclei are immunostained with DAPI (blue), endothelial cells with vonWillebrand factor (red) and podocytes with WT1 (green). Representative schematic of a glomerulus showing the location of parietal epithelial cells along the Bowman's capsule (B). *Adapted from (Romagnani, 2009)*.

In mouse models, studies have shown that the absence of either VEGF isoform during the embryonic and postnatal stage of life can lead to abnormal development of the glomerular filtration barrier (Eremina et al., 2003). Using electron microscopy it has become evident that VEGF is present within the glomerular filtration barrier specifically localised within the podocyte foot processes, glomerular basement membrane, luminal and abluminal surfaces of the endothelium (Eremina and Quaggin, 2004). Since VEGF- A expression continues during podocyte differentiation, this suggests that it plays a pivotal role in the maintenance or formation of the specialised features of the podocyte (Eremina and Quaggin, 2004).

Experimental studies have shown that the development of the glomerular filtration barrier requires tight regulation of VEGF within the glomerulus, suggesting dosage sensitivity for VEGF-A in glomerular capillary development (Eremina et al., 2003). Changes in VEGF levels can lead to dramatic consequences for glomerular development. A number of studies have shown that elevation of VEGF is associated with collapsing, diabetic glomerulopathy, diabetic retinopathy and microvascular end organ damage in diabetes (Eremina and Quaggin, 2004, Yang et al., 2003).

2.3 RENAL FUNCTION DURING NORMAL GESTATIONAL DEVELOPMENT

2.3.1 GLOMERULAR FUNCTION

During fetal life, the placenta is the major regulator of fetal fluid balance. At 9-10 weeks of gestation the fetus is able to produce urine, and this forms the majority of the amniotic fluid (Chevalier, 1996, Engle, 1986). *In utero*, renal blood flow and glomerular filtration rate (GFR) remain low until 20-30 weeks of gestation (Chevalier, 1996, Blackburn, 2003). During the early stage of pregnancy (10-20 weeks of gestation) the fetus receives only 5% of the total cardiac output (Rudolph et al., 1971). The renal blood flow then increases to 9% following term birth and 25%

of the total cardiac output in the adult (Rudolph et al., 1971, Lake and Booker, 2005). In response to being born, the newborn needs to rapidly adapt to the extrauterine environment and the kidney is required to independently control fluid and electrolyte levels. Following birth, there is a significant increase in mean arterial pressure, cardiac output and reduced renal vascular resistance. Since resistance of the afferent and efferent arterioles is a determinant of glomerular capillary pressure, the GFR also increases, thus increasing sodium reabsorption (Arant, 1987). GFR is used to measure kidney function, and can be estimated by calculating creatinine clearance (Stevens et al., 2006). Other measures of glomerular function include urine albumin and urine total protein levels. In the human kidney GFR does not reach normal adult values until 1-2 years of age (Rubin et al., 1949, Downing et al., 1992). Importantly, GFR *in utero* is strongly correlated with gestational age (Satlin et al., 2003). The GFR in the neonatal kidney is about 2 ml/min (Arant, 1978), and in the adult kidney it averages approximately 100 ml/min, in which the majority of filtered solutes and water are reabsorbed by the renal tubules.

2.3.2 TUBULAR FUNCTION

Following glomerular filtration, the filtrate from the glomerulus enter the renal tubules which are continuous with the Bowman's space of the renal corpuscles (Park and Kopan, 2016). The tubules of the nephron are responsible for reabsorbing up to 99% of the electrolytes and water and returning them to the blood stream (Zhuo and Li, 2013) and consist of: the proximal tubules, loop of Henle, the distal tubules and collecting tubules (Park and Kopan, 2016). The functional development of the proximal tubules involves rapid maturation of proximal tubular cells, and is said to continue until at least the 36^{th} – 39^{th} postconceptional week (Aperia et al., 1981). At birth there is a marked increase in GFR, which leads to a significant increase in the amount of sodium that is required to be filtered (Arant, 1987).

Hence, immediately after birth there is a rapid increase in tubular function and this leads to changes in tubular sodium reabsorption after birth and in early childhood (Lumbers, 1995). The sodium (Na^+) gradient across the epithelium is essential for the co-transport of Na^+ with bicarbonate, amino acids, glucose and other organic molecules (O'Callaghan, 2009). The reabsorption of filtered solutes occurs firstly in the proximal tubules (up to 70% of sodium reabsorption), followed by the distal tubules (approximately 25% of sodium reabsorption) and then collecting ducts (2-5% of sodium reabsorption) (Satlin et al., 2003, O'Callaghan, 2009). The active transport of sodium is completed by the time the filtrate enters the medullary region of the collecting tubules (Satlin et al., 2003). For this reason, the control of sodium balance is dependent on a balance between the function of the glomerulus and the different segments of the tubules. Hence, an important measure of tubular function is the calculation of fractional excretion of sodium (FE_{Na}), which is the percentage of sodium that is excreted and not taken up through tubular reabsorption (Gubhaju et al., 2014). In this regard, the fractional excretion of sodium in preterm neonates has been found to be inversely related to gestational age at birth, and to decrease with postnatal age (Bueva and Guignard, 1994, Gallini et al., 2000, Gubhaju et al., 2014). In the proximal tubules, an active transport process mediated by energy dependent mechanisms is required to drive sodium reabsorption (Zhuo and Li, 2013). In this regard, $Na^+ K^+$ ATPase has been recognised to play a crucial role in sodium transport across the proximal tubules (Feraille and Doucet, 2001). Increases in the activity of $Na^+ K^+$ ATPase transporters and Na^+/H^+ exchangers is particularly important for the growth and function of all segments of the tubules during the early postnatal period (Holtback and Aperia, 2003, Baum, 1992). As a result, there is marked tubular maturation and growth that occurs during the postnatal period.

In the human the proximal tubules have been shown to increase in size by 12-fold between birth and the age of 18 years (Fetterman et al., 1965). In addition to sodium excretion levels, urine β2-microglobulin levels are also good markers for assessing tubular function as the proximal tubule also functions to reabsorb filtered proteins. During fetal life, β2-microglobulin levels in the amniotic fluid have been noted to decrease with increasing renal development (Burghard et al., 1987). During the neonatal period, levels of β2-microglobulin have shown to be elevated compared to adults (Takieddine et al., 1983), with levels reaching a peak at the end of the first week of life and decreasing thereafter (Tsukahara et al., 1994).

3. PRETERM BIRTH AND ITS EFFECTS ON NEPHROGENESIS AND RENAL FUNCTION

3.1 THE EFFECTS OF PRETERM BIRTH ON NEPHROGENESIS

The nephrons are the functional units of the kidney and importantly, nephrogenesis (the formation of nephrons) is usually not completed until late in gestation (approximately 32 to 36 weeks gestation) (Hinchliffe et al., 1991). Hence, the majority of preterm infants are born at a time when nephrogenesis is still ongoing. Over the past decade there have been a number of studies looking at the effect of preterm birth on nephrogenesis in the kidney. In the first of these studies, Rodriguez et al. (Rodriguez et al., 2004) reported a reduced number of glomerular generations (thus implying reduced nephron endowment) in autopsied kidneys from babies that were born preterm compared to those born at term. However, in that study many of the preterm infants were also IUGR; hence, the interpretation of the findings is difficult, because it is well known that IUGR leads to reduced nephron endowment (see later section). Likewise, in another autopsy study the number of glomerular generations (a marker

of nephron number) was significantly reduced in preterm kidneys compared to terms (Faa et al., 2010). Concomitant with these studies, in our laboratory we have also conducted a number of studies looking at the effect of premature birth in immature preterm kidneys, both in a non-human primate model of preterm birth and in autopsied kidneys of preterm infants. These studies have convincingly shown that when nephrogenesis is ongoing in preterm infants at the time of birth, that nephrogenesis continues after birth; new nephrons are formed in the extra-uterine environment (Gubhaju et al., 2009, Sutherland et al., 2011). In the baboon studies, where the timing of nephrogenesis is similar to the human, we have shown that the kidneys are significantly larger in the preterm neonates, with a concomitant decrease in glomerular density, but nephron number was in the normal range (thus implying changes in tubular growth) (Gubhaju et al., 2009). Of concern, however, there was a high proportion of abnormal glomeruli (up to 18% in some kidneys) in the outer renal cortex in some of the preterm neonates. The abnormal glomeruli exhibited a shrunken immature glomerulus and an enlarged cystic Bowman's space. Similarly, in studies conducted in autopsied kidneys from infants born preterm (Sutherland et al., 2011), there was also an increase in kidney weight relative to body weight (probably due to the increased postnatal functional demands) and importantly new glomerular generations were formed after birth. However, there was a reduced nephrogenic zone width and a reduction in the proportion of glomeruli in the most immature stages (vesicle, comma-shaped, S-shaped and capillary loop stages), when compared to gestational age-matched controls, suggesting that cessation of nephrogenesis may be accelerated, and nephrogenic potential adversely impacted upon. To date, there have been no studies that have looked at exactly when nephrogenesis ceases in the preterm infant relative to gestational age-matched infants.

Alarmingly, as seen in the preterm baboon kidneys, there was a high proportion of abnormal glomeruli (with shrunken glomerular capillary tuft and an enlarged cystic Bowman's space) in the outer renal cortex (up to 13% of glomeruli) in some of the preterm human kidneys (Sutherland et al., 2011). Given the severity of these glomerular abnormalities it is unlikely that these glomeruli will ever be functional. Hence, these findings suggest that in these preterm infants the endowment of functional nephrons is adversely impacted upon by preterm birth, thereby likely affecting renal function both in the early postnatal period and later in life. To date, the causes of the glomerular abnormalities in the preterm kidneys are unknown. Given that not all preterm kidneys exhibit abnormal glomeruli, it is likely that it may be factors in the intrauterine environment (that lead to preterm delivery) that have adversely impacted upon the developing glomeruli or alternatively, it may be factors in the extra- uterine environment (haemodynamic and in the postnatal clinical care) that have led to these glomerular abnormalities. In addition,there may be intrauterine factors and/or extra-uterine factors that adversely impact on nephrogenesis without inflicting glomerular pathologies. Importantly, impairments in nephron development can lead to impairments in renal functional capacity.

3.2 THE EFFECT OF PRETERM BIRTH ON RENAL FUNCTION

As we are now aware, in the case of preterm infants, they are delivered at a time when nephrogenesis is often ongoing. In preterm neonates GFR is very low at birth, and does not rise as rapidly as full term infants during the neonatal period (Aperia et al., 1981, Bueva and Guignard, 1994). As expected, GFR has shown to increase more rapidly after 34 weeks' gestation (Arant, 1978, Satlin et al., 2003) which coincides with the timing of the completion of nephrogenesis. Numerous studies have shown that preterm birth can lead to a high incidence of renal dysfunction in the neonate and under severe circumstances this can lead to renal failure (Stapleton et al., 1987, Choker and Gouyon, 2004).

The incidence of renal impairment in preterm infants is difficult to clearly define given that the kidneys are very immature at the time of birth. Hence, renal function is quite different in the preterm infant when compared to the term infant and many of these differences are due to immaturity rather than an underlying impairment. Certainly, both glomerular and tubular function are influenced by gestational age at birth and hence, it is difficult to establish whether the differences in renal function in preterm infants compared to term infants are solely due to underdevelopment of the nephrons or the result of injury in an immature kidney. During the first week after birth, GFR is significantly lower in preterm infants compared to term infants (Siegel and Oh, 1976, Finney et al., 2000, Schreuder et al., 2009) and it is positively correlated with gestational age at birth and postnatal age (Clark et al., 1989, Gordjani et al., 1998, Iacobelli et al., 2009). Likewise, creatinine clearance, one of the most commonly used estimates of GFR, is positively correlated with both gestational age and postnatal age (Ross et al., 1977, Fawer et al., 1979, Sulyok et al., 1979, Aperia et al., 1981, Clark et al., 1989, Wilkins, 1992, Bueva and Guignard, 1994, Gordjani et al., 1998, Gallini et al., 2000, Cuzzolin et al., 2006, Thayyil et al., 2008, Iacobelli et al., 2009, Gubhaju et al., 2014). In addition, preterm neonates excrete high amounts of sodium in the early neonatal period compared to term neonates, with the fractional excretion of sodium inversely correlated with gestational age and postnatal age (Galaske, 1986, Clark et al., 1989, Tsukahara et al., 1990, Fell et al., 1997, Awad et al., 2002, Gubhaju et al., 2014). Furthermore, several studies have reported low gestational age to be a strong predictor of tubular proteinuria early after birth (Clark et al., 1989, Awad et al., 2002, Ojala et al., 2006). To date, it remains unclear whether the observed proteinuria in preterm infants is a result of their renal immaturity or due to postnatal renal injury.

3.2.1 PROTEINURIA

The presence of high levels of protein in the urine is indicative of pathological proteinuria (urine total protein ≥500mg/l) and can be glomerular and/or tubular in origin. The presence of proteins with a high molecular weight (such as albumin) in the urine is indicative of a disruption in the integrity of the glomerular filtration barrier (Mathieson, 2004). Indeed, in animal models it has been shown that podocyte abnormalities are strongly linked to the development of proteinuria (Wharram et al., 2005, Fukuda et al., 2012). In addition, small amounts of albuminuria, known as microalbuminuria, are considered to be good predictors of cardiovascular and renal disease risks in adults (Eknoyan et al., 2003).

Alternatively, high levels of low molecular weight proteins (such as β2-microglobulin, alpha1-microglobulin, and retinol binding protein) are indicative of reduced reuptake by the proximal tubule cells (Tomlinson, 1992, Christensen and Birn, 2001). The occurrence of proteinuria in neonates is strongly linked to gestational age at birth, with studies in preterm infants reporting significantly greater albumin and β2-microglobulin concentrations over the first month of life in infants born <32 weeks' gestation, compared to neonates born >32 weeks' gestation (Tsukahara et al., 1994, Gubhaju et al., 2014).

In the adult, in circumstances of renal impairment, increased excretion of low molecular weight proteins which exceeds the absorptive capacity of the tubules is indicative of significant renal pathology (Christensen and Gburek, 2004, Ojala et al., 2006).

Proteinuria has been recognised as a strong predictor of progressive kidney disease and end stage kidney disease (Jafar et al., 2001, Taal and Brenner, 2007, Methven et al., 2010) and

amongst the Indigenous population overt proteinuria has shown to be a significant predictor of reduced GFR, renal failure, and natural death (Hoy et al., 2001). In addition, amongst Indigenous Australians, albuminuria strongly correlates with low birth weight, and increases dramatically with ageing (Hoy et al., 1998, Hoy et al., 1999).

3.2.2 ACUTE KIDNEY INJURY

Acute kidney injury (previously defined as acute renal failure) is reported to occur in 8% to 24% of preterm infants admitted to neonatal intensive care units (Stapleton et al., 1987, Hentschel et al., 1996). The mortality amongst these infants that are born <32 weeks' gestation has been reported to be as high as 30-60% (Andreoli, 2004). Acute kidney injury is defined as a sustained extreme decline in creatinine clearance; the initial clinical symptoms are a marked increase in serum creatinine and/or a sustained very low urine output (Bellomo et al., 2004, Mehta et al., 2007). The major risk factors for acute kidney injury are very low gestational age and low birth weight (Walker et al., 2011). Other factors that have been linked to acute kidney injury are: hypotension, hypoxia, sepsis, maternal and neonatal drug administration (NSAIDs, indomethacin, antibiotics and vasopressors), a low apgar score, intraventricular haemorrhage (grade III and IV), necrotising enterocolitis, patent ductus arteriosus, respiratory distress syndrome, clinical interventions (intubation at birth), catheterisation, phototherapy, and mechanical ventilation (Stapleton et al., 1987, Cataldi et al., 2005, Walker et al., 2011). Of concern, mortality rates were reported to be significantly higher in neonates with renal dysfunction/renal failure (Walker et al., 2011).

In addition, to the short-term effects in the kidney, preterm birth is reported to influence long-term renal function (Keijzer-Veen et al., 2005, Rodriguez-Soriano et al., 2005, Iacobelli et al., 2007). For example, Rodriguez et al. (2005) found GFR to be significantly lower in children

ranging in age between 6.1 and 12.4 years who were born preterm compared to term-born controls, with evidence of renal injury such as defects in the tubular transport of phosphate (Rodriguez et al., 2005). Iacobelli et al. (2007), found that microalbuminaria was present in 8.3% of children examined that were born premature, ranging from 6-8 years of age. Similar findings were reported in a study of young adults; a lower GFR, higher serum creatinine and microalbuminaria was reported at 19 years of age in subjects born at <32 weeks' gestation (and also small for gestational age) (Keijzer-Veen et al., 2005). Furthermore, there is strong epidemiological evidence to link premature birth with the development of hypertension (Siewert-Delle and Ljungman, 1998, Irving et al., 2000, Kistner et al., 2000, Bonamy et al., 2005, Bonamy et al., 2007, Cooper et al., 2009) and increased cardiovascular risk during adulthood (Ciccone et al., 2011, Mercuro et al., 2013). This 'risk' may be further exacerbated in the presence of impaired renal function, which can itself lead to hypertension, and the possible development of cardiovascular disease in later life (Brenner and Chertow, 1994).

3.2.3 CHRONIC KIDNEY DISEASE AND END STAGE KIDNEY DISEASE

The incidence and prevalence of chronic kidney disease (CKD) and end-stage kidney disease (ESKD), also referred to as end stage renal disease, continues to increase worldwide (AIHW, 2010). CKD is increasingly recognised as a world health problem and can lead to kidney failure, cardiovascular disease and premature death (Levey et al., 2005). CKD is defined as kidney damage for greater than three months, as defined by structural or functional abnormalities of the kidney, with or without decreasing GFR that can lead to reduced GFR (Levey et al., 2005). In most cases, CKD is asymptotic by nature during the early period and therefore can lead to progression of renal disease indicated by impaired renal function (Jha and Modi, 2013). Currently, strong predictors of CKD in the adult are GFR below 60 mL/min/1.73 m^2 or

albuminuria (albumin: creatinine ratio greater than 3.4mg/mmol)(KDOQI, 2002, Wen et al., 2014). The incidence of CKD is around 10% worldwide (AIHW, 2010), with the progression of developing kidney disease being multifactorial; including; low birth weight (Hoy et al., 1998, Hoy et al., 1999, White et al., 2009), diabetes (AIHW, 2011), low socioeconomic conditions (Garcia-Garcia et al., 2015), obesity (Wang et al., 2008, Thomas et al., 2011, Mallamaci and Tripepi, 2013), sex (Berg, 2006, Neugarten et al., 2000), and ethnicity (Tarver-Carr et al., 2002, Hoy and McDonald, 2004, McDonald et al., 2003). Recent studies have suggested that antecedents of CKD may occur early in life, such as maternal factors (Komenda et al., 2014), low birth weight (White et al., 2009) and reduced nephron mass (Halbesma et al., 2011), rendering the kidneys more vulnerable to diabetes and hypertension. A recent meta- analysis of 31 studies found a 70% increase in relative risk of CKD with low birth weight, where low birthweight adults were 58% more likely to develop ESKD (White et al., 2009). In a long- term study, Hodgin et al. (2009), reported consistent findings of focal and segmental glomerulosclerosis, associated with glomerulomegaly, in renal biopsies of 6 adults born premature accompanied by low birth weight. Indeed, in both preterm birth and low birth weight can lead to glomerular hypertrophy; and glomerular hypertrophy has been reported to be independently associated with multiple CKD risk factors, and is a feature of multiple renal diseases (Hoy et al., 2008).This is not surprising given that approximately 90% of kidney diseases leading to ESKD originate in the glomeruli (Collins et al., 2014). In addition to low birth weight, in recent years it has become apparent that males have an increased prevalence of renal failure amongst individuals presenting with CKD, compared to females (Neugarten et al., 2000, Berg, 2006). However, in a retrospective study of over 2 million Norwegians, both males and females that were intrauterine growth restricted showed increased risk of developing ESKD (Vikse et al., 2008).

A plausible explanation for the association between low birth weight and CKD in later life may be an imbalance between apoptosis and cell proliferation in the developing kidney, followed by senescence and mitochondrial dysfunction that occur following IUGR (Hershkovitz et al., 2007).

It is certainly well recognised that Indigenous Australians show some of the highest rates of ESKD, where it is approximately 10 times more prevalent than in non-Indigenous Australians (ANZDATA, 2015). Similarly, African Americans have demonstrated higher rates of CKD and ESKD than non-African Americans (Tarver-Carr et al., 2002). The three main causes of ESKD amongst the Indigenous Australians include: induced diabetic nephropathy (number one cause of CKD and ESKD), primary glomerulonephritis, and hypertension (Stewart et al., 2004). It has been suggested that the high prevalence of albuminuria amongst the Indigenous population is linked to low nephron number and is related to low birth weight (Hoy and McDonald, 2004, McDonald et al., 2003). In this regard, it has been noted that Aborigines have 20% fewer nephrons than Australian whites from the same geographic region, as well as glomerular hypertrophy (Hoy et al., 2006). Together, these findings emphasise the importance of early intervention to help slow or prevent the progression of kidney disease in individuals that are susceptible from birth.

4. INTRAUTERINE GROWTH RESTRICTION (IUGR)

Intrauterine growth restriction (IUGR) is defined as body growth below the 10th percentile for gestational age. Many studies define IUGR using just the weight definition, and some studies use an absolute birth weight (such as less than 2500g) to indicate low birth weight regardless of appropriate weight for the gestational age of the infant.

4.1 GROWTH CHARTS

Recently, birth weight, length and head circumference have been recognised as good indicators of fetal and infant growth (Villar et al., 2015). Altered fetal growth can suggest adverse changes to the *in utero* environment. Growth charts are created using large cohorts, such as in the recent multicenter population-based study conducted between 2009 -2014 in 8 locations around the world (Villar et al., 2015). Studies such as this INTERGROWTH 21st Project, allow for reliable estimations of normal fetal and infant growth trajectories to be produced, therefore providing international standards for newborn weight, length and head circumference according to gestational age and sex. Currently, the weight of the fetus or infant is most commonly used to determine growth restriction. In this instance, fetuses or infants that have a body weight below the 10th percentile for their sex and age group are considered intrauterine growth restricted. Maternal and child health nurses currently use percentile charts from the World Health Organisation (Onis et al., 2006) for children between 0 and 24 months of age. However, recently, the INTERGROWTH 21st Project have addressed the limitations of the WHO growth charts, which assumed that the postnatal growth of preterm infants is similar to the growth of term infants during *in utero* life. Therefore, it is now recommended that international standards constructed by the INTERGROWTH 21st Project are to be used to evaluate growth of preterm infants until 64 weeks' postmenstrual age, after which the use of the WHO Child Growth Standards becomes appropriate (Villar et al., 2015).

4.2 TYPES OF INTRAUTERINE GROWTH RESTRICTION

Poor intrauterine growth leading to IUGR usually results as a consequence of reduced blood supply and/or nutrients to the fetus (Behrman et al., 1970, Lang et al., 2003, Kiserud, 2005, Cox

et al., 2009). There are two main types of IUGR – symmetrical and asymmetrical (Barker, 1995b, Cox et al., 2009).

4.2.1 SYMMETRICAL IUGR NEWBORNS

Newborns that are identified as having 'symmetrical IUGR' can also be regarded as having 'impaired growth potential' (Cox et al., 2009). Symmetrical IUGR occurs in the first few months of pregnancy and results from disorders affecting the fetal development during the stages of cell division, where one or more cycles of cell division is lost (Cox et al., 2009). Causes of symmetrical IUGR include rubella and cytomegalovirus infections, maternal malnutrition and smoking (Crane and Kopta, 1980). Subsequently reduced overall growth occurs, leading to a newborn that is small for its gestational age; all organs are equally affected, leading to a symmetrical appearance.

4.2.2 ASYMMETRICAL IUGR NEWBORNS

Asymmetrical IUGR occurs as a result of inadequate nutrition and oxygen supply to the fetus, usually as a result of acquired placental pathology; this predominantly occurs in the third trimester of pregnancy. As a consequence, there is a redistribution of the blood flow in the fetal circulation with blood supply directed to the brain at the expense of the other organs (Behrman et al., 1970). As a result, the brain maintains normal growth (termed 'brain sparing') whilst the viscera develop poorly (Barker, 1995a). The babies are born with a larger head to body ratio than that of non-IUGR newborns.

4.3 FACTORS THAT LEAD TO INTRAUTERINE GROWTH RESTRICTION

IUGR is multifactorial in origin with maternal race, economic status, diet and lifestyle (which can be interlinked) and complications of pregnancy all associated with induction of IUGR. IUGR is often a co-morbidity of preterm birth and it is linked to both spontaneous and assisted

premature deliveries. In many pregnancies, it is difficult to ascertain whether it is the underlying cause of the IUGR, or the poor *in utero* growth of the fetus that is the stimulus for spontaneous preterm delivery. Likewise, the developing kidney can be directly impacted upon by the factors leading to IUGR, or alternatively, it can be a direct corollary of the IUGR itself. Certainly, the general consensus of the findings from the literature would support the latter with IUGR (regardless of the underlying causes) linked to poor organ development in the fetus and concomitant impairment of kidney development (Hinchliffe et al., 1992, Manalich et al., 2000). In the next sections some of the common factors associated with IUGR are described, including their links with preterm delivery.

4.3.1 MATERNAL ETHNICITY / SOCIO-ECONOMIC STATUS

Maternal race has been linked with both premature delivery and IUGR (Fiscella, 1996, Schieve and Handler, 1996, Goldenberg et al., 2008). For example, in the USA, African and African American women have been shown to have a four times higher chance of delivering a premature newborn compared to other racial groups (Goldenberg et al., 2008). In addition, women from South Asia and the Indian subcontinent have very high rates of IUGR and low birth weight (Goldenberg et al., 2008), whereas, women from East Asia and Hispanic regions have been shown to have lower rates of premature delivery. In Australia, Indigenous Australians have a much higher frequency of IUGR and preterm delivery (approximately twice that of non-indigenous Australians) (Laws et al., 2010, Shah et al., 2011). It is important to note, that in many of these populations (where there is a high incidence of IUGR) there is also a low socioeconomic status. Hence, the underlying cause of the IUGR may be due to poor maternal nutrition, lifestyle insults and poor maternal health (all described below), rather than their ethnicity *per se*.

4.3.2 MATERNAL DIET

Malnutrition is a common cause of IUGR in developing countries (de Onis et al., 1998). It can result from under nutrition (inadequate food intake) and/or restriction of specific key nutrients in the diet. For example, data from the Dutch famine during World War II found that children born to mothers that had limited food available (less than 1000 calories per day) over the majority of their pregnancy gave birth to babies that were small for gestational age (Painter et al., 2005). In another large study conducted in 538 women who delivered at term, it was shown that reduced protein intake during pregnancy led to low birth weight in the neonates (Godfrey et al., 1996). Similarly, in rat studies, IUGR is consistently reported when rat dams are fed a low protein diet during pregnancy (Langley and Jackson, 1994, Merlet-Benichou et al., 1994, Desai et al., 1996, Zimanyi et al., 2006).

4.3.3 MATERNAL LIFESTYLE

Maternal behaviours such as smoking, high alcohol consumption, and ingesting illicit drugs have all been recognised as contributors to the risk of IUGR and premature birth (Lazzaroni et al., 1993, Burguet et al., 2004, Sokol et al., 2007, Jaddoe et al., 2008, O'Leary et al., 2009). Cigarette smoking has been reported to increase the risk of premature rupture of the membranes, pregnancy bleeding and pre-term labour. In addition, maternal smoking has been identified as a major cause of IUGR in developed countries, contributing to as many as 40% of all cases of IUGR (de Onis et al., 1998). Smoking causes vascular changes in the mother that can lead to placental insufficiency and hypoxia in the fetus (Maccani et al., 2010). It has also been associated with the down-regulation of important miRNAs of the placenta, leading to newborns that are small for gestational age (Maccani et al., 2010). Furthermore, nicotine found in cigarettes has been shown to pass through the placenta, thus exerting a direct negative effect

on the growth of the fetus (Maccani et al., 2010). Importantly, Dejmek et al. (2002) also showed that reduced birth weight in newborns of smoking mothers was dose-dependent (that is, dependent on the number of cigarettes smoked per day).

Consumption of alcohol and use of illicit drugs during pregnancy is also linked to increased risk of preterm birth. In a cohort of 3000 African American women, alcohol and cocaine use was found to be associated with extreme preterm birth (Sokol et al., 2007). Of particular concern, a study by O'Leary et al. (2009), found that moderate ingestion of alcohol consumption (only during the first trimester of pregnancy) was associated with pre-term birth. In Australia, the high rate of preterm birth in the Indigenous community is thought to be attributed to high rates of tobacco, alcohol and drug use in pregnant women (Shah et al., 2011).

4.3.4 PLACENTAL INSUFFICIENCY/ABRUPTION

The placenta is a vital organ that develops specifically during pregnancy to support the growth of the developing fetus. The role of the placenta is to supply the fetus with an adequate amount of nutrients and oxygen for normal fetal growth. In developed countries the most common cause of IUGR is placental insufficiency (Henriksen and Clausen, 2002, Schreuder et al., 2006), and it is also strongly linked with preterm birth (Baschat et al., 2007). Placental insufficiency occurs when the placenta does not develop normally and thus it is unable to adequately support the developing baby. It is usually caused by reduced uterine artery blood flow (uteroplacental insufficiency) (Henriksen and Clausen, 2002).

Placental abruption occurs in late gestation and is a serious condition where the placenta partially, or completely, separates from the lining of the uterus; the effects on the developing fetus depend on the severity of the abruption (Ananth et al., 2005). The full separation of the placenta from the uterus lining can lead to *in utero* death and subsequent stillbirth, if the fetus

is not delivered at the time of abruption. When there is partial placental separation the fetus is growth restricted and preterm birth will often ensue (spontaneous or assisted).

4.3.5 PRE-ECLAMPSIA

Pre-eclampsia is pregnancy-associated hypertension (Lyell et al., 2003); it is a multi-system disorder which affects approximately 8% of pregnancies (Duley, 2009). It occurs when placentation is abnormal, which can cause the mother to experience intravascular coagulation, bleeding and organ failure (hepatic and renal) following poor perfusion. These complications subside with the delivery of the fetus. Severe pre-eclampsia can lead to maternal death and thus, it is a major cause of assisted preterm birth (Sibai et al., 2005). In addition, pre-eclampsia during pregnancy is a major risk factor for IUGR (Churchill et al., 1997, Walker et al., 1998) as it usually results in placental insufficiency. Higher rates of pre-eclampsia are seen amongst women with pre-existing hypertension, diabetes mellitus or previous history of pre-eclampsia (Sibai, 2003). Of concern, there has been an increased incidence of pre-eclampsia in developing countries over recent years (Lopez-Jaramillo et al., 2005).

4.3.6 MULTIPLE BIRTHS

Pregnancies with multiple fetuses exhibit a higher risk of placental dysfunction and placental insufficiency. This results in the slowed growth rate of twins (or higher order multiples) during late gestation in comparison to the singleton growth rate (Smith et al., 2001). The incidence of multiple births is increasing and this is largely attributed to the increase in availability of infertility treatment, such as ovulation induction (Keith and Oleszczuk, 1999, Fauser et al., 2005). Monochorionic twins (identical twins that share one placenta) have a much greater chance of being born IUGR than dichorionic twins (twins that do not share the same placenta)

(Lynch et al., 2003, Chang et al., 2011b). Discordant growth results from an unequal distribution of uteroplacental blood flow to the fetuses (Breathnach and Malone, 2012).

4.3.7 FETAL HYPOXIA

Intrauterine hypoxia occurs when the fetus is deprived of an adequate supply of oxygen and is a common cause of IUGR, specifically asymmetrical IUGR (Hutter et al., 2010). As reviewed in Hutter et al.(2010) and Giussani et al. (2016), there are three main causes of intrauterine hypoxia including: *(1)* Preplacental hypoxia (mother and fetus are hypoxic), usually a result of factors such as high altitude, maternal smoking, maternal respiratory disease, pre-existing maternal cardiovascular disease and/or maternal anemia, infections, and chronic inflammation. *(2)* Utero-placental hypoxia (normal maternal oxygenation but utero-placental circulation is impaired), usually caused by preeclampsia (discussed above), placental insufficiencies, and prolonged membrane rupture. *(3)* Post-placental hypoxia (fetus is hypoxic), usually a result of reduced uterine artery flow or fetal cardiac structural abnormalities/progressive cardiac failure. Initially, the fetus is able to adapt to a hypoxic environment by increasing the blood supply to the brain and upper body and decreasing the perfusion of the kidneys and other peripheral organs and extremities (Giussani et al., 1993, Wollmann, 1998, Cataldi et al., 2005, Giussani, 2016). During late gestation the fetus is able to accommodate an acute hypoxic environment by decreasing fetal breathing, and heart rate, in order to reduce the required oxygen consumption (Giussani et al., 1995, Boddy et al., 1974). Exposure to chronic hypoxia (in the fetal rat), however, was found to cause reduced kidney weight and greater vasoconstriction in the fetus, resulting in altered renal interlobar artery function (Tang et al., 2015). Furthermore, in a lamb model, fetal hypoxemia caused a marked reduction in renal blood flow, filtration fraction, and mean arterial blood pressure in very

preterm and near-term fetuses (Robillard et al., 1980). In addition, fetuses exhibiting hypoxemia demonstrated a marked increase in fractional excretion of sodium, with significant increases in creatinine clearance in the very preterm hypoxic fetuses, indicative of glomerular and tubular injury (Robillard et al., 1980).

5. IUGR ADVERSELY IMPACTS ON NEPHRON ENDOWMENT AT BIRTH

It is now well established that IUGR, regardless of the etiological origins (many of these described above), can adversely impact on the number of nephrons formed within the developing kidney. Indeed, there are many experimental studies that have shown that when IUGR is induced by maternal dietary manipulations, or by the induction of placental insufficiency, that nephron number is reduced in the offspring (Lucas et al., 1997, Louey et al., 2000, Ozaki et al., 2001, Mitchell et al., 2004, Moritz et al., 2009). In general, nephron endowment at birth is directly proportional to kidney size (Zohdi et al., 2007, Gubhaju et al., 2009, Sutherland et al., 2009), so in the case of the IUGR infant the reduction in body size at birth is accompanied by a decrease in kidney size and in the number of nephrons. In support of this concept, in a study of autopsied human kidneys there was found to be a linear relationship between the number of glomeruli (and therefore nephrons) and birth weight in full term neonates (Holland, 1993); neonates below the 10^{th} percentile of birth weight had 30% fewer glomeruli than the neonates with birth weights above the 10^{th} percentile (Holland, 1993).

However, it is important to note that the timing of the growth insult during gestation is important. If the growth restriction occurs late in gestation, when nephrogenesis is already complete or close to completion, the number of nephrons formed within the kidney will not be

affected by the IUGR; although, birth weight will be significantly reduced. For example, in a study performed in our laboratory (Mitchell et al., 2004), placental insufficiency was experimentally induced in fetal lambs late in gestation (from 120-140 days gestation; term is 147 days) at a time when nephrogenesis was nearing completion. This study revealed a significant decrease in body weight and kidney weight in response to IUGR compared to appropriately grown lambs. However, nephron endowment in the IUGR lambs was not different to the control lambs. In contrast, IUGR caused by twinning led to a significant reduction in nephron endowment (Mitchell et al., 2004).

5.1 CONSEQUENCES OF LOW NEPHRON ENDOWMENT

To date, both animal and human studies have shown that there are many causes of low nephron number, including: low birth weight, female gender, exposure to gestational diabetes, ethnicity, and small kidney size (Luyckx et al., 2011).

Low nephron number can lead to long-term adverse consequences including impaired renal function, glomerulosclerosis, further nephron loss, and hypertension (Ingelfinger, 2008, Luyckx et al., 2011). Initially Brenner and Chow (1994), proposed that a reduced nephron endowment, due to low birth weight, causes a reduced renal filtration surface area, leading to reduced capacity for sodium excretion. Subsequently, compensatory glomerular hypertrophy and hyperfiltration occurs, thereby leading to further loss of nephrons and eventually glomerulosclerosis which can lead to development of hypertension. Keller et al. (2003) was one of the first studies conducted in humans to link reduced nephron number with hypertension. This study reported in hypertensive subjects, that the glomerular number was reduced by ~47%, with 133% higher mean glomerular volume compared to non-hypertensive subjects. Following this, Hughson and colleagues (2006) documented that white American males with

hypertension also exhibited reduced nephron numbers. These findings were further validated by Hoy and colleagues (2005, 2006), that reported Indigenous Australians with hypertension had 30% fewer nephrons, and subsequently a 25% increase in glomerular volume, compared to non-hypertensive Indigenous Australians. However, to date it remains unclear how preterm birth and intrauterine growth restriction affect nephron number and subsequently renal function during the early neonatal period. Of concern, nephron deficits may be further exacerbated when causes of low nephron number, such as preterm birth and IUGR, occur concomitantly. In this instance, preterm infants that are growth restricted can experience rapid catch-up growth during the neonatal period that can further render the kidney vulnerable to secondary insults. For example, exposure to obesity, high salt diets, and diabetes (Boubred et al., 2007) in later life. In addition to the development of hypertension, low nephron number has been linked to increased salt sensitivity, proteinuria, reduced renal functional reserve, and eventual glomerulosclerosis, chronic kidney disease and end stage kidney disease (Luyckx et al., 2011).

FOCUS OF THIS THESIS

The focus of this thesis was to examine the effects of preterm birth on kidney development and function during the early postnatal period. Furthermore, this thesis explores the effects of IUGR on the developing human kidney and the combined impact of preterm birth and IUGR during the early neonatal period.

AIMS

The specific aims of each of the studies in this thesis are listed below:

Chapter 2: To conduct a comprehensive histological examination of the developing human kidney from 20 weeks in gestation until term, the developmental period when the majority of nephrons are formed.

Chapter 3: To examine the effect of moderate preterm birth and/or mechanical ventilation on glomerular capillary growth.

Chapter 4: To explore the effects of preterm birth on the cellular composition of the glomerulus and in particular glomerular podocyte endowment.

Chapter 5: To characterise the effects of IUGR on nephrogenesis in the developing human kidney from mid gestation through to term (the gestational period when the majority of nephrons are formed) and secondly, to assess the impact on renal growth and function in the first month of life when IUGR is combined with preterm birth.

Chapter 6: To compare the levels of renal injury in the immediate period after birth and after a month of life in Indigenous and non-Indigenous Australian preterm and term infants.

REFERENCES

ABDEL-HADY, H., NASEF, N., SHABAAN, A. E. & NOUR, I. 2013. Patent ductus arteriosus in preterm infants: do we have the right answers? *Biomed Res Int,* 2013, 676192.

ABRAHAMSON, D. & ROBERT, B. 2003. Derivation and differentiation of glomerular endothelial cells. *Nephrol Dial Transplant,* 18, vi2-7.

ABRAHAMSON, D. R. 1991. Glomerulogenesis in the developing kidney. *Semin Nephrol,* 11, 375-89.

ABRAHAMSON, D. R. & WANG, R. 2003. Development of the glomerular capillary and its basement membrane. *In:* VIZE, P. D., WOOLF, A. S. & BARD, J. B. L. (eds.) *The Kidney: From Normal Development to Congenital Disease.* Sydney: Academic Press.

AHA & AAP 2006. 2005 American Heart Association (AHA) guidelines for cardiopulmonary resuscitation (CPR) and emergency cardiovascular care (ECC) of pediatric and neonatal patients: neonatal resuscitation guidelines. *Pediatrics,* 117, e1029-38.

AIHW 2010. Australia's health 2010. Australia's health no. 12. Cat. no. AUS 122. Canberra: AIHW.

AIHW 2011. Australia's welfare 2011. Australia's welfare no. 10. Cat. no. AUS 142. Canberra: AIHW.

AL-AWQATI, Q. & GOLDBERG, M. R. 1998. Architectural patterns in branching morphogenesis in the kidney. *Kidney Int,* 54, 1832-42.

ALDER, J., FINK, N., BITZER, J., HOSLI, I. & HOLZGREVE, W. 2007. Depression and anxiety during pregnancy: a risk factor for obstetric, fetal and neonatal outcome? A critical review of the literature. *J Matern Fetal Neonatal Med,* 20, 189-209.

ALLEN, M. C., DONOHUE, P. K. & DUSMAN, A. E. 1993. The limit of viability--neonatal outcome of infants born at 22 to 25 weeks' gestation. *N Engl J Med,* 329, 1597-601.

ALY, H., HAMMAD, T., NADA, A., MOHAMED, M., BATHGATE, S. & EL-MOHANDES, A. 2010. Maternal obesity, associated complications and risk of prematurity. *J Perinatol,* 30, 447-51.

ANANTH, C. V., SAVITZ, D. A., LUTHER, E. R. & BOWES, W. A., JR. 1997. Preeclampsia and preterm birth subtypes in Nova Scotia, 1986 to 1992. *Am J Perinatol,* 14, 17-23.

ANANTH, C. V., SMULIAN, J. C., SRINIVAS, N., GETAHUN, D. & SALIHU, H. M. 2005. Risk of infant mortality among twins in relation to placental abruption: contributions of preterm birth and restricted fetal growth. *Twin Res Hum Genet,* 8, 524-31.

ANDREOLI, S. 2004. Acute Renal Failure in the Newborn. *Seminars in Perinatology,* 28, 112-123.

ANZDATA 2015. Chapter 12: Indigenous People and End Stage Kidney Disease. Australia and New Zealand Dialysis and transplant Registry, Adelaide, Australia. *ANZDATA Registry. 37th Report*.

APERIA, A., BROBERGER, O., ELINDER, G., HERIN, P. & ZETTERSTROM, R. 1981. Postnatal development of renal function in pre-term and full-term infants. *Acta Paediatr Scand,* 70, 183-7.

ARANT, B. S., JR. 1978. Developmental patterns of renal functional maturation compared in the human neonate. *J Pediatr,* 92, 705-12.

ARANT, B. S., JR. 1987. Postnatal development of renal function during the first year of life. *Pediatr Nephrol,* 1, 308-13.

AWAD, H., EL-SAFTY, I., EL-BARBARY, M. & IMAM, S. 2002. Evaluation of renal glomerular and tubular functional and structural integrity in neonates. *Am J Med Sci,* 324, 261-6.

BARKER, D. 1995a. Fetal origins of coronary heart disease. *Br Med J,* 311, 171-4.

BARKER, D. J. 1995b. Intrauterine programming of adult disease. *Mol Med Today,* 1, 418-23.

BARLOW, P., LEJEUNE, B., PUISSANT, F., ENGLERT, Y., VANRYSSELBERGE, M., DEGUELDRE, M., VEKEMANS, M. & LEROY, F. 1988. Early-Pregnancy Loss and Obstetrical Risk after Invitro Fertilization and Embryo Replacement. *Human Reproduction,* 3, 671-675.

BARROS, F. C., PAPAGEORGHIOU, A. T., VICTORA, C. G., NOBLE, J. A., PANG, R., IAMS, J., CHEIKH ISMAIL, L., GOLDENBERG, R. L., LAMBERT, A., KRAMER, M. S., CARVALHO, M., CONDE-AGUDELO, A., JAFFER, Y. A., BERTINO, E., GRAVETT, M. G., ALTMAN, D. G., OHUMA, E. O., PURWAR, M., FREDERICK, I. O., BHUTTA, Z. A., KENNEDY, S. H., VILLAR, J., INTERNATIONAL, F. & NEWBORN GROWTH CONSORTIUM FOR THE 21ST, C. 2015. The distribution of clinical phenotypes of preterm birth syndrome: implications for prevention. *JAMA Pediatr,* 169, 220-9.

BASCHAT, A. A., COSMI, E., BILARDO, C. M., WOLF, H., BERG, C., RIGANO, S., GERMER, U., MOYANO, D., TURAN, S., HARTUNG, J., BHIDE, A., MULLER, T., BOWER, S., NICOLAIDES,

K. H., THILAGANATHAN, B., GEMBRUCH, U., FERRAZZI, E., HECHER, K., GALAN, H. L. & HARMAN, C. R. 2007. Predictors of neonatal outcome in early-onset placental dysfunction. *Obstet Gynecol,* 109, 253-61.

BAUM, M. 1992. Developmental changes in rabbit juxtamedullary proximal convoluted tubule acidification. *Pediatr Res,* 31, 411-4.

BECK, S., WOJDYLA, D., SAY, L., BETRAN, A. P., MERIALDI, M., REQUEJO, J. H., RUBENS, C., MENON, R. & VAN LOOK, P. F. 2010. The worldwide incidence of preterm birth: a systematic review of maternal mortality and morbidity. *Bull World Health Organ,* 88, 31-8.

BEEN, J. V., LUGTENBERG, M. J., SMETS, E., VAN SCHAYCK, C. P., KRAMER, B. W., MOMMERS, M. & SHEIKH, A. 2014. Preterm Birth and Childhood Wheezing Disorders: A Systematic Review and Meta-Analysis. *Plos Medicine,* 11.

BEHRMAN, R. E., LEES, M. H., PETERSON, E. N., DE LANNOY, C. W. & SEEDS, A. E. 1970. Distribution of the circulation in the normal and asphyxiated fetal primate. *Am J Obstet Gynecol,* 108, 956-69.

BELLOMO, R., RONCO, C., KELLUM, J. A., MEHTA, R. L. & PALEVSKY, P. 2004. 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, R204-12.

BENSLEY, J. G., DE MATTEO, R., HARDING, R. & BLACK, M. J. 2012. Preterm birth with antenatal corticosteroid administration has injurious and persistent effects on the structure and composition of the aorta and pulmonary artery. *Pediatr Res,* 71, 150-5.

BENSLEY, J. G., STACY, V. K., DE MATTEO, R., HARDING, R. & BLACK, M. J. 2010. Cardiac remodelling as a result of pre-term birth: implications for future cardiovascular disease. *Eur Heart J,* 31, 2058-66.

BERG, U. B. 2006. Differences in decline in GFR with age between males and females. Reference data on clearances of inulin and PAH in potential kidney donors. *Nephrol Dial Transplant,* 21, 2577-82.

BLACKBURN, S. 2003. Renal system and fluid and electrolyte homeostasis. *Maternal, fetal and neonatal physiology.* 2 ed.: Saunders.

BLACKBURN, S. 2007. *Maternal, Fetal, and Neonatal Physiology - A Clinical Perspective,* St Louis Missouri USA, Elsevier.

BLENCOWE, H., COUSENS, S., OESTERGAARD, M. Z., CHOU, D., MOLLER, A. B., NARWAL, R., ADLER, A., VERA GARCIA, C., ROHDE, S., SAY, L. & LAWN, J. E. 2012. National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications. *Lancet,* 379, 2162-72.

BLONDEL, B., KOGAN, M. D., ALEXANDER, G. R., DATTANI, N., KRAMER, M. S., MACFARLANE, A. & WEN, S. W. 2002. The impact of the increasing number of multiple births on the rates of preterm birth and low birthweight: an international study. *Am J Public Health,* 92, 1323-30.

BODDY, K., JONES, C. T., MANTELL, C., RATCLIFFE, J. G. & ROBINSON, J. S. 1974. Changes in plasma ACTH and corticosteroid of the maternal and fetal sheep during hypoxia. *Endocrinology,* 94, 588-91.

BONAMY, A. K., BENDITO, A., MARTIN, H., ANDOLF, E., SEDIN, G. & NORMAN, M. 2005. Preterm birth contributes to increased vascular resistance and higher blood pressure in adolescent girls. *Pediatr Res,* 58, 845-9.

BONAMY, A. K., MARTIN, H., JORNESKOG, G. & NORMAN, M. 2007. Lower skin capillary density, normal endothelial function and higher blood pressure in children born preterm. *J Intern Med,* 262, 635-42.

BOUBRED, F., BUFFAT, C., FEUERSTEIN, J. M., DANIEL, L., TSIMARATOS, M., OLIVER, C., LELIEVRE-PEGORIER, M. & SIMEONI, U. 2007. Effects of early postnatal hypernutrition on nephron number and long-term renal function and structure in rats. *Am J Physiol Renal Physiol,* 293, F1944-9.

BREATHNACH, F. M. & MALONE, F. D. 2012. Fetal growth disorders in twin gestations. *Semin Perinatol,* 36, 175-81.

BRENNER, B. M. & CHERTOW, G. M. 1994. Congenital oligonephropathy and the etiology of adult hypertension and progressive renal injury. *Am J Kidney Dis,* 23, 171-5.

BROWN, J. S., JR., ADERA, T. & MASHO, S. W. 2008. Previous abortion and the risk of low birth weight and preterm births. *J Epidemiol Community Health,* 62, 16-22.

BUCHMAYER, S. M., SPAREN, P. & CNATTINGIUS, S. 2004. Previous pregnancy loss: risks related to severity of preterm delivery. *Am J Obstet Gynecol,* 191, 1225-31.

BUEVA, A. & GUIGNARD, J. P. 1994. Renal function in preterm neonates. *Pediatr Res,* 36, 572-7.

BURGHARD, R., PALLACKS, R., GORDJANI, N., LEITITIS, J. U., HACKELOER, B. J. & BRANDIS, M. 1987. Microproteins in amniotic fluid as an index of changes in fetal renal function during development. *Pediatric Nephrology,* 1, 574-80.

BURGUET, A., KAMINSKI, M., ABRAHAM-LERAT, L., SCHAAL, J. P., CAMBONIE, G., FRESSON, J., GRANDJEAN, H., TRUFFERT, P., MARPEAU, L., VOYER, M., ROZE, J. C., TREISSER, A. & LARROQUE, B. 2004. The complex relationship between smoking in pregnancy and very preterm delivery. Results of the Epipage study. *Bjog,* 111, 258-65.

BURKE, G. L., EVANS, G. W., RILEY, W. A., SHARRETT, A. R., HOWARD, G., BARNES, R. W., ROSAMOND, W., CROW, R. S., RAUTAHARJU, P. M. & HEISS, G. 1995. Arterial-Wall Thickness Is Associated with Prevalent Cardiovascular-Disease in Middle-Aged Adults - the Atherosclerosis Risk in Communities (Aric) Study. *Stroke,* 26, 386-391.

CARROLL, T. J., PARK, J. S., HAYASHI, S., MAJUMDAR, A. & MCMAHON, A. P. 2005. Wnt9b plays a central role in the regulation of mesenchymal to epithelial transitions underlying organogenesis of the mammalian urogenital system. *Developmental Cell,* 9, 283-292.

CATALDI, L., LEONE, R., MORETTI, U., DE MITRI, B., FANOS, V., RUGGERI, L., SABATINO, G., TORCASIO, F., ZANARDO, V., ATTARDO, G., RICCOBENE, F., MARTANO, C., BENINI, D. & CUZZOLIN, L. 2005. Potential risk factors for the development of acute renal failure in preterm newborn infants: a case-control study. *Arch Dis Child Fetal Neonatal Ed,* 90, F514-9.

CHANG, Y. L., CHANG, S. D., CHAO, A. S., WANG, C. N., WANG, T. H. & CHENG, P. J. 2011a. The Relationships of Umbilical Venous Volume Flow, Birthweight and Placental Share in Monochorionic Twin Pregnancies With and Without Selective Intrauterine Growth Restriction. *Twin Research and Human Genetics,* 14, 192-197.

CHANG, Y. L., CHANG, S. D., CHAO, A. S., WANG, C. N., WANG, T. H. & CHENG, P. J. 2011b. The relationships of umbilical venous volume flow, birthweight and placental share in

monochorionic twin pregnancies with and without selective intrauterine growth restriction. *Twin Res Hum Genet,* 14, 192-7.

CHEVALIER, R. 1996. Developmental renal physiology of the low birth weight pre-term newborn. *J Urol,* 156, 714-9.

CHI, X., MICHOS, O., SHAKYA, R., RICCIO, P., ENOMOTO, H., LICHT, J. D., ASAI, N., TAKAHASHI, M., OHGAMI, N., KATO, M., MENDELSOHN, C. & COSTANTINI, F. 2009. Ret-dependent cell rearrangements in the Wolffian duct epithelium initiate ureteric bud morphogenesis. *Dev Cell,* 17, 199-209.

CHOKER, G. & GOUYON, J. B. 2004. Diagnosis of Acute Renal Failure in Very Preterm Infants. *Biol Neonate,* 86, 212-216.

CHRISTENSEN, E. I. & BIRN, H. 2001. Megalin and cubilin: synergistic endocytic receptors in renal proximal tubule. *Am J Physiol Renal Physiol,* 280, F562-73.

CHRISTENSEN, E. I. & GBUREK, J. 2004. Protein reabsorption in renal proximal tubule-function and dysfunction in kidney pathophysiology. *Pediatric Nephrology,* 19, 714-21.

CHURCHILL, D., PERRY, I. J. & BEEVERS, D. G. 1997. Ambulatory blood pressure in pregnancy and fetal growth. *Lancet,* 349, 7-10.

CICCONE, M. M., SCICCHITANO, P., ZITO, A., GESUALDO, M., SASSARA, M., CALDERONI, G., DI MAURO, F., LADISA, G., DI MAURO, A. & LAFORGIA, N. 2011. Different functional cardiac characteristics observed in term/preterm neonates by echocardiography and tissue doppler imaging. *Early Hum Dev,* 87, 555-8.

CLARK, P. M., BRYANT, T. N., HALL, M. A., LOWES, J. A. & ROWE, D. J. 1989. Neonatal renal function assessment. *Arch Dis Child,* 64, 1264-9.

COLLINS, A. J., FOLEY, R. N., CHAVERS, B., GILBERTSON, D., HERZOG, C., ISHANI, A., JOHANSEN, K., KASISKE, B. L., KUTNER, N., LIU, J., ST PETER, W., GUO, H., HU, Y., KATS, A., LI, S., LI, S., MALONEY, J., ROBERTS, T., SKEANS, M., SNYDER, J., SOLID, C., THOMPSON, B., WEINHANDL, E., XIONG, H., YUSUF, A., ZAUN, D., ARKO, C., CHEN, S. C., DANIELS, F., EBBEN, J., FRAZIER, E., JOHNSON, R., SHEETS, D., WANG, X., FORREST, B., BERRINI, D., CONSTANTINI, E., EVERSON, S., EGGERS, P. & AGODOA, L. 2014. US Renal Data System 2013 Annual Data Report. *Am J Kidney Dis,* 63, A7.

COOLEN, J., KABAYASHI, K., WONG, K., MAYES, D. C., BOTT, N. & DEMIANCZUK, N. 2010. Influence of oligohydramnios on preterm premature rupture of the membranes at 30 to 36 weeks' gestation. *J Obstet Gynaecol Can,* 32, 1030-4.

COOPER, R., ATHERTON, K. & POWER, C. 2009. Gestational age and risk factors for cardiovascular disease: evidence from the 1958 British birth cohort followed to mid-life. *Int J Epidemiol,* 38, 235-44.

COSTANTINI, F. 2006. Renal branching morphogenesis: concepts, questions, and recent advances. *Differentiation,* 74, 402-421.

COSTANTINI, F. & KOPAN, R. 2010. Patterning a complex organ: branching morphogenesis and nephron segmentation in kidney development. *Dev Cell,* 18, 698-712.

COX, B., KOTLYAR, M., EVANGELOU, A. I., IGNATCHENKO, V., IGNATCHENKO, A., WHITELEY, K., JURISICA, I., ADAMSON, S. L., ROSSANT, J. & KISLINGER, T. 2009. Comparative systems biology of human and mouse as a tool to guide the modeling of human placental pathology. *Mol Syst Biol,* 5, 279.

CRANE, J. P. & KOPTA, M. M. 1980. Comparative Newborn Anthropometric Data in Symmetric Versus Asymmetric Intrauterine Growth-Retardation. *American Journal of Obstetrics and Gynecology,* 138, 518-522.

CRUMP, C., WINKLEBY, M. A., SUNDQUIST, K. & SUNDQUIST, J. 2011. Risk of hypertension among young adults who were born preterm: a Swedish national study of 636,000 births. *Am J Epidemiol,* 173, 797-803.

CULLEN-MCEWEN, L., SUTHERLAND, M. R. & BLACK, M. J. 2016. Chapter 3 - The Human Kidney: Parallels in Structure, Spatial Development, and Timing of Nephrogenesis A2 - Little, Melissa H. *Kidney Development, Disease, Repair and Regeneration.* San Diego: Academic Press.

CUZZOLIN, L., FANOS, V., PINNA, B., DI MARZIO, M., PERIN, M., TRAMONTOZZI, P., TONETTO, P. & CATALDI, L. 2006. Postnatal renal function in preterm newborns: a role of diseases, drugs and therapeutic interventions. *Pediatric Nephrology,* 21, 931-938.

DANNEVIG, I., DALE, H. C., LIESTOL, K. & LINDEMANN, R. 2005. Blood pressure in the neonate: three non-invasive oscillometric pressure monitors compared with invasively measured blood pressure. *Acta Paediatr,* 94, 191-6.

DAVIS, P. H., DAWSON, J. D., RILEY, W. A. & LAUER, R. M. 2001. Carotid intimal-medial thickness is related to cardiovascular risk factors measured from childhood through middle age: The Muscatine Study. *Circulation,* 104, 2815-9.

DE FELICE, C., TOTI, P., LAURINI, R. N., STUMPO, M., PICCIOLINI, E., TODROS, T., TANGANELLI, P., BUONOCORE, G. & BRACCI, R. 2001. Early neonatal brain injury in histologic chorioamnionitis. *J Pediatr,* 138, 101-4.

DE ONIS, M., BLOSSNER, M. & VILLAR, J. 1998. Levels and patterns of intrauterine growth retardation in developing countries. *Eur J Clin Nutr,* 52 Suppl 1, S5-15.

DEJMEK, J., SOLANSK Y, I., PODRAZILOVA, K. & SRAM, R. J. 2002. The exposure of nonsmoking and smoking mothers to environmental tobacco smoke during different gestational phases and fetal growth. *Environ Health Perspect,* 110, 601-6.

DESAI, M., CROWTHER, N. J., LUCAS, A. & HALES, C. N. 1996. Organ-selective growth in the offspring of protein-restricted mothers. *Br J Nutr,* 76, 591-603.

DICE, J. E. & BHATIA, J. 2007. Patent ductus arteriosus: an overview. *J Pediatr Pharmacol Ther,* 12, 138-46.

DOWNING, G., EGELHOFF, J. & DAILY, D. 1992. Kidney function in very low birth weight infants with furosemide-related renal calcifications at ages 1 to 2 years. *J Pediatr,* 120, 599-604.

DOYLE, L. W. 2008. Cardiopulmonary outcomes of extreme prematurity. *Semin Perinatol,* 32, 28-34.

DRESSLER, G. R. 2006. The cellular basis of kidney development. *Annu Rev Cell Dev Biol,* 22, 509-29.

DRESSLER, G. R. 2009. The specification and maintenance of renal cell types by epigenetic factors. *Organogenesis,* 5, 73-82.

DULEY, L. 2009. The Global Impact of Pre-eclampsia and Eclampsia. *Seminars in Perinatology,* 33, 130-137.

EKNOYAN, G., HOSTETTER, T., BAKRIS, G. L., HEBERT, L., LEVEY, A. S., PARVING, H. H., STEFFES, M. W. & TOTO, R. 2003. Proteinuria and other markers of chronic kidney disease: a position statement of the national kidney foundation (NKF) and the national institute of diabetes and digestive and kidney diseases (NIDDK). *Am J Kidney Dis,* 42, 617-22.

ENGLE, W. 1986. Development of fetal and neonatal renal function. *Seminars in Perinatology,* 10, 113-124.

EREMINA, V. & QUAGGIN, S. E. 2004. The role of VEGF-A in glomerular development and function. *Curr Opin Nephrol Hypertens,* 13, 9-15.

EREMINA, V., SOOD, M., HAIGH, J., NAGY, A., LAJOIE, G., FERRARA, N., GERBER, H. P., KIKKAWA, Y., MINER, J. H. & QUAGGIN, S. E. 2003. Glomerular-specific alterations of VEGF-A expression lead to distinct congenital and acquired renal diseases. *J Clin Invest,* 111, 707-16.

ERIKSSON, U. & ALITALO, K. 1999. Structure, expression and receptor-binding properties of novel vascular endothelial growth factors. *Curr Top Microbiol Immunol,* 237, 41-57.

FAA, G., GEROSA, C., FANNI, D., NEMOLATO, S., LOCCI, A., CABRAS, T., MARINELLI, V., PUDDU, M., ZAFFANELLO, M., MONGA, G. & FANOS, V. 2010. Marked interindividual variability in renal maturation of preterm infants: lessons from autopsy. *J Matern Fetal Neonatal Med,* 23 Suppl 3, 129-33.

FAROOQI, A., HAGGLOF, B., SEDIN, G., GOTHEFORS, L. & SERENIUS, F. 2006. Chronic conditions, functional limitations, and special health care needs in 10 to 12-year-old children born at 23 to 25 weeks' gestation in the 1990s: a Swedish national perspective follow-up study. *Pediatrics,* 118, e1466-77.

FAUSER, B. C. J. M., DEVROEY, P. & MACKLON, N. S. 2005. Mulltiple birth resulting from ovarian stimulation for subfertility treatment. *Lancet,* 365, 1807-1816.

FAWER, C. L., TORRADO, A. & GUIGNARD, J. P. 1979. Maturation of renal function in full-term and premature neonates. *Helv Paediatr Acta,* 34, 11-21.

FELL, J. M., THAKKAR, H., NEWMAN, D. J. & PRICE, C. P. 1997. Measurement of albumin and low molecular weight proteins in the urine of newborn infants using a cotton wool ball collection method. *Acta Paediatr,* 86, 518-22.

FERAILLE, E. & DOUCET, A. 2001. Sodium-potassium-adenosinetriphosphatase-dependent sodium transport in the kidney: Hormonal control. *Physiological Reviews,* 81, 345-418.

FETTERMAN, G. H., SHUPLOCK, N. A., PHILIPP, F. J. & GREGG, H. S. 1965. The Growth and Maturation of Human Glomeruli and Proximal Convolutions from Term to Adulthood: Studies by Microdissection. *Pediatrics,* 35, 601-19.

FINER, N., SAUGSTAD, O., VENTO, M., BARRINGTON, K., DAVIS, P., DUARA, S., LEONE, T., LUI, K., MARTIN, R., MORLEY, C., RABI, Y. & RICH, W. 2010. Use of oxygen for resuscitation of the extremely low birth weight infant. *Pediatrics,* 125, 389-91.

FINNEY, H., NEWMAN, D. J., THAKKAR, H., FELL, J. M. & PRICE, C. P. 2000. Reference ranges for plasma cystatin C and creatinine measurements in premature infants, neonates, and older children. *Arch Dis Child,* 82, 71-5.

FISCELLA, K. 1996. Racial disparities in preterm births. The role of urogenital infections. *Public Health Rep,* 111, 104-13.

FISCHER, B. & BAVISTER, B. D. 1993. Oxygen tension in the oviduct and uterus of rhesus monkeys, hamsters and rabbits. *J Reprod Fertil,* 99, 673-9.

FRASER, J., WALLS, M. & MCGUIRE, W. 2004. Respiratory complications of preterm birth. *Bmj,* 329, 962-5.

FUKUDA, A., CHOWDHURY, M. A., VENKATAREDDY, M. P., WANG, S. Q., NISHIZONO, R., SUZUKI, T., WICKMAN, L. T., WIGGINS, J. E., MUCHAYI, T., FINGAR, D., SHEDDEN, K. A., INOKI, K. & WIGGINS, R. C. 2012. Growth-dependent podocyte failure causes glomerulosclerosis. *J Am Soc Nephrol,* 23, 1351-63.

GALASKE, R. G. 1986. Renal functional maturation: renal handling of proteins by mature and immature newborns. *Eur J Pediatr,* 145, 368-71.

GALLINI, F., MAGGIO, L., ROMAGNOLI, C., MARROCCO, G. & TORTOROLO, G. 2000. Progression of renal function in preterm neonates with gestational age < or = 32 weeks. *Pediatr Nephrol,* 15, 119-24.

GAO, Y. & RAJ, J. U. 2010. Regulation of the pulmonary circulation in the fetus and newborn. *Physiol Rev,* 90, 1291-335.

GARCIA-GARCIA, G., JHA, V., TAO LI, P. K., GARCIA-GARCIA, G., COUSER, W. G., ERK, T., ZAKHAROVA, E., SEGANTINI, L., SHAY, P., RIELLA, M. C., OSAFO, C., DUPUIS, S. & KERNAHAN, C. 2015. Chronic kidney disease (CKD) in disadvantaged populations. *Clin Kidney J,* 8, 3-6.

GAVIN, A. R., HOLZMAN, C., SIEFERT, K. & TIAN, Y. 2009. Maternal depressive symptoms, depression, and psychiatric medication use in relation to risk of preterm delivery. *Womens Health Issues,* 19, 325-34.

GEORGAS, K., RUMBALLE, B., VALERIUS, M. T., CHIU, H. S., THIAGARAJAN, R. D., LESIEUR, E., ARONOW, B. J., BRUNSKILL, E. W., COMBES, A. N., TANG, D., TAYLOR, D., GRIMMOND, S. M., POTTER, S. S., MCMAHON, A. P. & LITTLE, M. H. 2009. Analysis of early nephron patterning reveals a role for distal RV proliferation in fusion to the ureteric tip via a cap mesenchyme-derived connecting segment. *Dev Biol,* 332, 273-86.

GILBERT, S. F. 2006. *Developmental Biology, Eighth Edition. Chapter 14: Paraxial and Intermediate Mesoderm,* Sunderland, MA, USA, Sinauer Associates Inc. pp. 461.

GIUSSANI, D. A. 2016. The fetal brain sparing response to hypoxia: physiological mechanisms. *J Physiol,* 594, 1215-30.

GIUSSANI, D. A., BENNET, L., SFERRUZZI-PERRI, A. N., VAUGHAN, O. R. & FOWDEN, A. L. 2016. Hypoxia, fetal and neonatal physiology: 100 years on from Sir Joseph Barcroft. *J Physiol,* 594, 1105-11.

GIUSSANI, D. A., MOORE, P. J., BENNET, L., SPENCER, J. A. & HANSON, M. A. 1995. Alpha 1- and alpha 2-adrenoreceptor actions of phentolamine and prazosin on breathing movements in fetal sheep in utero. *J Physiol,* 486 (Pt 1), 249-55.

GIUSSANI, D. A., SPENCER, J. A., MOORE, P. J., BENNET, L. & HANSON, M. A. 1993. Afferent and efferent components of the cardiovascular reflex responses to acute hypoxia in term fetal sheep. *J Physiol,* 461, 431-49.

GODFREY, K., ROBINSON, S., BARKER, D. J., OSMOND, C. & COX, V. 1996. Maternal nutrition in early and late pregnancy in relation to placental and fetal growth. *BMJ,* 312, 410-4.

GOLDENBERG, R. L. & CULHANE, J. F. 2006. Preterm birth and periodontal disease. *N Engl J Med,* 355, 1925-7.

GOLDENBERG, R. L. & CULHANE, J. F. 2007. Low birth weight in the United States. *Am J Clin Nutr,* 85, 584S-590S.

GOLDENBERG, R. L., CULHANE, J. F., IAMS, J. D. & ROMERO, R. 2008. Epidemiology and causes of preterm birth. *Lancet,* 371, 75-84.

GOLDENBERG, R. L., HAUTH, J. C. & ANDREWS, W. W. 2000. Intrauterine infection and preterm delivery. *N Engl J Med,* 342, 1500-7.

GORDJANI, N., BURGHARD, R., LEITITIS, J. & BRANDIS, M. 1998. Serum creatinine and creatinine clearance in healthy neonates and prematures during the first 10 days of life. *Eur J Pediatr,* 148, 143-145.

GUBHAJU, L., SUTHERLAND, M. R. & BLACK, M. J. 2011. Preterm birth and the kidney: implications for long-term renal health. *Reprod Sci,* 18, 322-33.

GUBHAJU, L., SUTHERLAND, M. R., HORNE, R. S., MEDHURST, A., KENT, A. L., RAMSDEN, A., MOORE, L., SINGH, G., HOY, W. E. & BLACK, M. J. 2014. Assessment of renal functional maturation and injury in preterm neonates during the first month of life. *Am J Physiol Renal Physiol,* 307, F149-58.

GUBHAJU, L., SUTHERLAND, M. R., YODER, B. A., ZULLI, A., BERTRAM, J. F. & BLACK, M. J. 2009. Is nephrogenesis affected by preterm birth? Studies in a non-human primate model. *Am J Physiol Renal Physiol,* 297, F1668-77.

HALBESMA, N., JANSEN, D. F., HEYMANS, M. W., STOLK, R. P., DE JONG, P. E., GANSEVOORT, R. T. & GRP, P. S. 2011. Development and Validation of a General Population Renal Risk Score. *Clinical Journal of the American Society of Nephrology,* 6, 1731-1738.

HENDLER, I., GOLDENBERG, R. L., MERCER, B. M., IAMS, J. D., MEIS, P. J., MOAWAD, A. H., MACPHERSON, C. A., CARITIS, S. N., MIODOVNIK, M., MENARD, K. M., THURNAU, G. R. & SOROKIN, Y. 2005. The Preterm Prediction study: Association between maternal body mass index and spontaneous and indicated preterm birth. *American Journal of Obstetrics and Gynecology,* 192, 882-886.

HENRIKSEN, T. & CLAUSEN, T. 2002. The fetal origins hypothesis: placental insufficiency and inheritance versus maternal malnutrition in well-nourished populations. *Acta Obstet Gynecol Scand,* 81, 112-4.

HENTSCHEL, R., LODIGE, B. & BULLA, M. 1996. Renal insufficiency in the neonatal period. *Clin Nephrol* 46, 54-58.

HERNANDEZ, J. S., TWICKLER, D. M., MCINTIRE, D. D. & DASHE, J. S. 2012. Hydramnios in twin gestations. *Obstet Gynecol,* 120, 759-65.

HERSHKOVITZ, D., BURBEA, Z., SKORECKI, K. & BRENNER, B. M. 2007. Fetal programming of adult kidney disease: cellular and molecular mechanisms. *Clin J Am Soc Nephrol,* 2, 334-42.

HILL, G. A., BRYAN, S., HERBERT, C. M., SHAH, D. M. & WENTZ, A. C. 1990. Complications of Pregnancy in Infertile Couples - Routine Treatment Versus Assisted Reproduction. *Obstetrics and Gynecology,* 75, 790-794.

HINCHLIFFE, S. A., LYNCH, M. R., SARGENT, P. H., HOWARD, C. V. & VAN VELZEN, D. 1992. The effect of intrauterine growth retardation on the development of renal nephrons. *Br J Obstet Gynaecol,* 99, 296-301.

HINCHLIFFE, S. A., SARGENT, P. H., HOWARD, C. V., CHAN, Y. F. & VAN VELZEN, D. 1991. Human intrauterine renal growth expressed in absolute number of glomeruli assessed by the disector method and Cavalieri principle. *Lab Invest,* 64, 777-84.

HODGIN, J. B., RASOULPOUR, M., MARKOWITZ, G. S. & D'AGATI, V. D. 2009. Very low birth weight is a risk factor for secondary focal segmental glomerulosclerosis. *Clin J Am Soc Nephrol,* 4, 71-6.

HOLLAND, P. 1993. Placental insufficiency and its effect on the fetus and adult disease. *Lancet,* 341, 827.

HOLTBACK, U. & APERIA, A. C. 2003. Molecular determinants of sodium and water balance during early human development. *Semin Neonatol,* 8, 291-9.

HOY, W. & MCDONALD, S. P. 2004. Albuminuria: marker or target in indigenous populations. *Kidney Int Suppl*, S25-31.

HOY, W. E., BERTRAM, J. F., DENTON, R. D., ZIMANYI, M., SAMUEL, T. & HUGHSON, M. D. 2008. Nephron number, glomerular volume, renal disease and hypertension. *Curr Opin Nephrol Hypertens,* 17, 258-65.

HOY, W. E., HUGHSON, M. D., BERTRAM, J. F., DOUGLAS-DENTON, R. & AMANN, K. 2005. Nephron number, hypertension, renal disease, and renal failure. *J Am Soc Nephrol,* 16, 2557-64.

HOY, W. E., HUGHSON, M. D., SINGH, G. R., DOUGLAS-DENTON, R. & BERTRAM, J. F. 2006. Reduced nephron number and glomerulomegaly in Australian Aborigines: a group at high risk for renal disease and hypertension. *Kidney Int,* 70, 104-10.

HOY, W. E., REES, M., KILE, E., MATHEWS, J. D., MCCREDIE, D. A., PUGSLEY, D. J. & WANG, Z. 1998. Low birthweight and renal disease in Australian aborigines. *The Lancet,* 352, 1826-1827.

HOY, W. E., REES, M., KILE, E., MATHEWS, J. D. & WANG, Z. 1999. A new dimension to the Barker hypothesis: Low birth weight and susceptibility to renal disease. *Kidney International,* 56, 1072-1077.

HOY, W. E., WANG, Z., VANBUYNDER, P., BAKER, P. R., MCDONALD, S. M. & MATHEWS, J. D. 2001. The natural history of renal disease in Australian Aborigines. Part 2. Albuminuria predicts natural death and renal failure. *Kidney Int,* 60, 249-56.

HUGHSON, M. D., DOUGLAS-DENTON, R., BERTRAM, J. F. & HOY, W. E. 2006. Hypertension, glomerular number, and birth weight in African Americans and white subjects in the southeastern United States. *Kidney Int,* 69, 671-678.

HUTTER, D., KINGDOM, J. & JAEGGI, E. 2010. Causes and mechanisms of intrauterine hypoxia and its impact on the fetal cardiovascular system: a review. *Int J Pediatr,* 2010, 401323.

IACOBELLI, S., BONSANTE, F., FERDINUS, C., LABENNE, M. & GOUYON, J. B. 2009. Factors affecting postnatal changes in serum creatinine in preterm infants with gestational age <32 weeks. *J Perinatol,* 29, 232-6.

IACOBELLI, S., LOPRIENO, S., BONSANTE, F., LATORRE, G., ESPOSITO, L. & GOUYON, J. B. 2007. Renal function in early childhood in very low birthweight infants. *Am J Perinatol,* 24, 587-92.

INGELFINGER, J. R. 2008. Disparities in renal endowment: causes and consequences. *Adv Chronic Kidney Dis,* 15, 107-14.

IRVING, R., BELTON, N., ELTON, R. & WALKER, B. 2000. Adult cardiovascular risk factors in premature babies. *Lancet,* 355, 2135-6.

JADDOE, V. W., DE RIDDER, M. A., VAN DEN ELZEN, A. P., HOFMAN, A., UITERWAAL, C. S. & WITTEMAN, J. C. 2008. Maternal smoking in pregnancy is associated with cholesterol development in the offspring: A 27-years follow-up study. *Atherosclerosis,* 196, 42-8.

JAFAR, T. H., STARK, P. C., SCHMID, C. H., LANDA, M., MASCHIO, G., MARCANTONI, C., DE JONG, P. E., DE ZEEUW, D., SHAHINFAR, S., RUGGENENTI, P., REMUZZI, G., LEVEY, A. S., INHIBITION, A. S. G. A.-C. E. & PROGRESSION OF RENAL, D. 2001. Proteinuria as a modifiable risk factor for the progression of non-diabetic renal disease. *Kidney Int,* 60, 1131-40.

JHA, V. & MODI, G. K. 2013. Cardiovascular disease: The price of a QALY--cost-effectiveness of statins in CKD. *Nat Rev Nephrol,* 9, 377-9.

JOHANSSON, S., ILIADOU, A., BERGVALL, N., TUVEMO, T., NORMAN, M. & CNATTINGIUS, S. 2005. Risk of high blood pressure among young men increases with the degree of immaturity at birth. *Circulation,* 112, 3430-6.

KALLAPUR, S. G. & IKEGAMI, M. 2006. Physiological consequences of intrauterine insults. *Paediatr Respir Rev,* 7, 110-6.

KAMLIN, C. O., O'DONNELL, C. P., DAVIS, P. G. & MORLEY, C. J. 2006. Oxygen saturation in healthy infants immediately after birth. *J Pediatr,* 148, 585-9.

KDOQI 2002. National Kidney Foundation. KDOQI Clinical Practice Guidelines for Chronic Kidney Disease: Evaluation, Classification and Stratification. *Am J Kidney Dis,* 39.

KEIJZER-VEEN, M., SCHREVEL, M., FINKEN, M., DEKKER, F., NAUTA, J., HILLE, E., FROLICH, M. & VAN DER HEIJDEN, B. 2005. Microalbuminuria and lower glomerular filtration rate at young adult age in subjects born very premature and after intrauterine growth retardation. *Journal of the American Society of Nephrology* 16, 2762-8.

KEITH, L. & OLESZCZUK, J. J. 1999. Iatrogenic multiple birth, multiple pregnancy and assisted reproductive technologies. *Int J Gynaecol Obstet,* 64, 11-25.

KELLER, G., ZIMMER, G., MALL, G., RITZ, E. & AMANN, K. 2003. Nephron number in patients with primary hypertension. *New England Journal of Medicine,* 348, 101-108.

KERKHOF, G. F., BREUKHOVEN, P. E., LEUNISSEN, R. W., WILLEMSEN, R. H. & HOKKEN-KOELEGA, A. C. 2012. Does preterm birth influence cardiovascular risk in early adulthood? *J Pediatr,* 161, 390-396 e1.

KIKKAWA, Y., VIRTANEN, I. & MINER, J. H. 2003. Mesangial cells organize the glomerular capillaries by adhering to the G domain of laminin alpha5 in the glomerular basement membrane. *J Cell Biol,* 161, 187-96.

KISERUD, T. 2005. Physiology of the fetal circulation. *Semin Fetal Neonatal Med,* 10, 493-503.

KISTNER, A., CELSI, G., VANPEE, M. & JACOBSON, S. H. 2000. Increased blood pressure but normal renal function in adult women born preterm. *Pediatr Nephrol,* 15, 215-20.

KOBAYASHI, A., KWAN, K. M., CARROLL, T. J., MCMAHON, A. P., MENDELSOHN, C. L. & BEHRINGER, R. R. 2005. Distinct and sequential tissue-specific activities of the LIM-class homeobox gene Lim1 for tubular morphogenesis during kidney development. *Development,* 132, 2809-23.

KOBAYASHI, A., VALERIUS, M. T., MUGFORD, J. W., CARROLL, T. J., SELF, M., OLIVER, G. & MCMAHON, A. P. 2008. Six2 defines and regulates a multipotent self-renewing nephron progenitor population throughout mammalian kidney development. *Cell Stem Cell,* 3, 169-81.

KOMENDA, P., RIGATTO, C. & TANGRI, N. 2014. Estimated glomerular filtration rate and albuminuria: diagnosis, staging, and prognosis. *Curr Opin Nephrol Hypertens,* 23, 251-7.

KREIDBERG, J. A., SARIOLA, H., LORING, J. M., MAEDA, M., PELLETIER, J., HOUSMAN, D. & JAENISCH, R. 1993. Wt-1 Is Required for Early Kidney Development. *Cell,* 74, 679-691.

KUTZ, P., HORSCH, S., KUHN, L. & ROLL, C. 2009. Single-centre vs. population-based outcome data of extremely preterm infants at the limits of viability. *Acta Paediatr,* 98, 1451-5.

LAKE, C. L. & BOOKER, P. D. 2005. Pediatric cardiac anesthesia / editors, Carol L. Lake, Peter D. Booker. *Philadelphia Lippincott Williams & Wilkins*.

LANG, U., BAKER, R. S., BRAEMS, G., ZYGMUNT, M., KUNZEL, W. & CLARK, K. E. 2003. Uterine blood flow--a determinant of fetal growth. *Eur J Obstet Gynecol Reprod Biol,* 110 Suppl 1, S55-61.

LANGLEY, S. C. & JACKSON, A. A. 1994. Increased systolic blood pressure in adult rats induced by fetal exposure to maternal low protein diets. *Clinical Science (Lond),* 86, 217-222.

LAWS, P. J., LI, Z. & SULLIVAN, E. A. 2010. Australia's mothers and babies 2008. *Perinatal statistics series no. 24. Cat. no. PER 50 Canberra: AIHW*.

LAZZARONI, F., BONASSI, S., MAGNANI, M., CALVI, A., REPETTO, E., SERRA, F., PODESTA, F. & PEARCE, N. 1993. Moderate maternal drinking and outcome of pregnancy. *Eur J Epidemiol,* 9, 599-606.

LEVEY, A. S., ECKARDT, K. U., TSUKAMOTO, Y., LEVIN, A., CORESH, J., ROSSERT, J., DE ZEEUW, D., HOSTETTER, T. H., LAMEIRE, N. & EKNOYAN, G. 2005. Definition and classification of chronic kidney disease: a position statement from Kidney Disease: Improving Global Outcomes (KDIGO). *Kidney Int,* 67, 2089-100.

LITTLE, M. H. 2006. Regrow or repair: potential regenerative therapies for the kidney. *J Am Soc Nephrol,* 17, 2390-401.

LOPEZ-JARAMILLO, P., GARCIA, R. G. & LOPEZ, M. 2005. Preventing pregnancy-induced hypertension: are there regional differences for this global problem? *J Hypertens,* 23, 1121-9.

LOUEY, S., COCK, M. L., STEVENSON, K. M. & HARDING, R. 2000. Placental insufficiency and fetal growth restriction lead to postnatal hypotension and altered postnatal growth in sheep. *Pediatr Res,* 48, 808-14.

LUCAS, S. R., COSTA SILVA, V. L., MIRAGLIA, S. M. & ZALADEK GIL, F. 1997. Functional and morphometric evaluation of offspring kidney after intrauterine undernutrition. *Pediatr Nephrol,* 11, 719-23.

LUDWIG, K. S. & LANDMANN, L. 2005. Early development of the human mesonephros. *Anat Embryol (Berl),* 209, 439-47.

LUMBERS, E. R. 1995. Development of renal function in the fetus: a review. *Reprod Fertil Dev,* 7, 415-26.

LUYCKX, V. A., SHUKHA, K. & BRENNER, B. M. 2011. Low nephron number and its clinical consequences. *Rambam Maimonides Med J,* 2, e0061.

LYELL, D. J., LAMBERT-MESSERLIAN, G. M. & GIUDICE, L. C. 2003. Prenatal screening, epidemiology, diagnosis, and management of preeclampsia. *Clin Lab Med,* 23, 413-42.

LYNCH, A., MCDUFFIE, R., STEPHENS, J., MURPHY, J., FABER, K. & ORLEANS, M. 2003. The contribution of assisted conception, chorionicity and other risk factors to very low birthweight in a twin cohort. *BJOG,* 110, 405-10.

MACCANI, M. A., AVISSAR-WHITING, M., BANISTER, C. E., MCGONNIGAL, B., PADBURY, J. F. & MARSIT, C. J. 2010. Maternal cigarette smoking during pregnancy is associated with downregulation of miR-16, miR-21, and miR-146a in the placenta. *Epigenetics,* 5, 583-9.

MADAN, J., CHEN, M., GOODMAN, E., DAVIS, J., ALLAN, W. & DAMMANN, O. 2010. Maternal obesity, gestational hypertension, and preterm delivery. *J Matern Fetal Neonatal Med,* 23, 82-8.

MALLAMACI, F. & TRIPEPI, G. 2013. Blood pressure variability in chronic kidney disease patients. *Blood Purif,* 36, 58-62.

MANALICH, R., REYES, L., HERRERA, M., MELENDI, C. & FUNDORA, I. 2000. Relationship between weight at birth and the number and size of renal glomeruli in humans: a histomorphometric study. *Kidney Int,* 58, 770-3.

MANCUSO, M. S., FIGUEROA, D., SZYCHOWSKI, J. M., PADEN, M. M. & OWEN, J. 2011. Midtrimester bacterial vaginosis and cervical length in women at risk for preterm birth. *Am J Obstet Gynecol,* 204, 342 e1-5.

MANUCK, T., YI, J., VARNER, M., HOLMGREN, C. & ESPLIN, S. 2011. Rate of Cervical Length Change and the Prediction of Recurrent Preterm Birth. *Reproductive Sciences,* 18, 265a-265a.

MARTIN, J. A., HAMILTON, B. E., VENTURA, S. J., OSTERMAN, M. J., KIRMEYER, S., MATHEWS, T. J. & WILSON, E. C. 2011. Births: final data for 2009. *Natl Vital Stat Rep,* 60, 1-70.

MATHEWS, T. J., MININO, A. M., OSTERMAN, M. J., STROBINO, D. M. & GUYER, B. 2011. Annual summary of vital statistics: 2008. *Pediatrics,* 127, 146-57.

MATHIESON, P. 2004. The cellular basis of albuminuria. *Clinical Science,* 107, 533 - 538.

MCDONALD, S. P., MAGUIRE, G. P. & HOY, W. E. 2003. Renal function and cardiovascular risk markers in a remote Australian Aboriginal community. *Nephrol Dial Transplant,* 18, 1555-61.

MEHTA, R. L., KELLUM, J. A., SHAH, S. V., MOLITORIS, B. A., RONCO, C., WARNOCK, D. G. & LEVIN, A. 2007. Acute Kidney Injury Network: report of an initiative to improve outcomes in acute kidney injury. *Crit Care,* 11, R31.

MERCER, B. M., MACPHERSON, C. A., GOLDENBERG, R. L., GOEPFERT, A. R., HAUGUEL-DE MOUZON, S., VARNER, M. W., IAMS, J. D., MEIS, P. J., MOAWAD, A. H., MIODOVNIK, M., CARITIS, S. N., VAN DORSTEN, J. P., SOROKIN, Y., THURNAU, G. R., SPONG, C. Y. & NETWORK, N. M.-F. M. U. 2006. Are women with recurrent spontaneous preterm births different from those without such history? *Am J Obstet Gynecol,* 194, 1176-84; discussion 1184-5.

MERCURO, G., BASSAREO, P. P., FLORE, G., FANOS, V., DENTAMARO, I., SCICCHITANO, P., LAFORGIA, N. & CICCONE, M. M. 2013. Prematurity and low weight at birth as new conditions predisposing to an increased cardiovascular risk. *Eur J Prev Cardiol,* 20, 357-67.

MERLET-BENICHOU, C., GILBERT, T., MUFFAT-JOLY, M., LELIEVRE-PEGORIER, M. & LEROY, B. 1994. Intrauterine growth retardation leads to a permanent nephron deficit in the rat. *Pediatric Nephrology,* 8, 175-80.

METHVEN, S., MACGREGOR, M. S., TRAYNOR, J. P., O'REILLY, D. S. & DEIGHAN, C. J. 2010. Assessing proteinuria in chronic kidney disease: protein-creatinine ratio versus albumin-creatinine ratio. *Nephrol Dial Transplant,* 25, 2991-6.

MICHOS, O. 2009. Kidney development: from ureteric bud formation to branching morphogenesis. *Curr Opin Genet Dev,* 19, 484-90.

MIKKOLA, K., RITARI, N., TOMMISKA, V., SALOKORPI, T., LEHTONEN, L., TAMMELA, O., PAAKKONEN, L., OLSEN, P., KORKMAN, M. & FELLMAN, V. 2005. Neurodevelopmental outcome at 5 years of age of a national cohort of extremely low birth weight infants who were born in 1996-1997. *Pediatrics,* 116, 1391-400.

MINER, J. H. 2011. Organogenesis of the kidney glomerulus: focus on the glomerular basement membrane. *Organogenesis,* 7, 75-82.

MINER, J. H., PATTON, B. L., LENTZ, S. I., GILBERT, D. J., SNIDER, W. D., JENKINS, N. A., COPELAND, N. G. & SANES, J. R. 1997. The laminin alpha chains: expression, developmental transitions, and chromosomal locations of alpha1-5, identification of heterotrimeric laminins 8-11, and cloning of a novel alpha3 isoform. *J Cell Biol,* 137, 685-701.

MITCHELL, E. K., LOUEY, S., COCK, M. L., HARDING, R. & BLACK, M. J. 2004. Nephron endowment and filtration surface area in the kidney after growth restriction of fetal sheep. *Pediatr Res,* 55, 769-73.

MOORE, T., HENNESSY, E. M., MYLES, J., JOHNSON, S. J., DRAPER, E. S., COSTELOE, K. L. & MARLOW, N. 2012. Neurological and developmental outcome in extremely preterm children born in England in 1995 and 2006: the EPICure studies. *British Medical Journal,* 345.

MORITZ, K. M., MAZZUCA, M. Q., SIEBEL, A. L., MIBUS, A., ARENA, D., TARE, M., OWENS, J. A. & WLODEK, M. E. 2009. Uteroplacental insufficiency causes a nephron deficit, modest renal insufficiency but no hypertension with ageing in female rats. *J Physiol,* 587, 2635-46.

MOSS, T. J. M. 2006. Respiratory consequences of preterm birth. *Clin Exp Pharmacol Physiol,* 33, 280-284.

MWANSA-KAMBAFWILE, J., COUSENS, S., HANSEN, T. & LAWN, J. E. 2010. Antenatal steroids in preterm labour for the prevention of neonatal deaths due to complications of preterm birth. *Int J Epidemiol,* 39 Suppl 1, i122-33.

NEUGARTEN, J., ACHARYA, A. & SILBIGER, S. R. 2000. Effect of gender on the progression of nondiabetic renal disease: a meta-analysis. *J Am Soc Nephrol,* 11, 319-29.

NORMAN, M. 2010. Preterm birth--an emerging risk factor for adult hypertension? *Semin Perinatol,* 34, 183-7.

NORTHWAY, W., ROSAN, R. & PORTER, D. 1967. Pulmonary disease following respiratory therapy of hyaline-membrane disease. Bronchopulmonary dysplasia. *N Engl J Med,* 276, 357-68.

O'CALLAGHAN, C. A. 2009. *The Renal System at a Glance,* Chichester, UK, Wiley-Blackwell.

O'LEARY, C. M., NASSAR, N., KURINCZUK, J. J. & BOWER, C. 2009. The effect of maternal alcohol consumption on fetal growth and preterm birth. *Bjog,* 116, 390-400.

OHLSSON, A. & SHAH, S. S. 2011. Ibuprofen for the prevention of patent ductus arteriosus in preterm and/or low birth weight infants. *Cochrane Database of Systematic Reviews*.

OJALA, R., ALA-HOUHALA, M., HARMOINEN, A. P., LUUKKAALA, T., UOTILA, J. & TAMMELA, O. 2006. Tubular proteinuria in pre-term and full-term infants. *Pediatr Nephrol,* 21, 68- 73.

OLIVER, J. 1968. Nephrons and Kidneys; A Quantitative Study of Developmental and Evolutionary Mammalian Renal Architectonics. *New York: Hoeber Medical Division Harper & Row*.

ONIS, M., ONYANGO, A., BORGHI, E., SIYAM, A., PINOL, A., GARZA, C., DE ONIS, M., MARTINES, J., MARTORELL, R., VICTORA, C. G., BHAN, M. K., DE ONIS, M., MARTINES, J., ONYANGO, A., PINOL, A., VICTORA, C. G., ARAUJO, C. L., LARTEY, A., OWUSU, W. B., BHAN, M. K., BHANDARI, N., NORUM, K. R., AA, G. E., MOHAMED, A. J., DEWEY, K. G., GARZA, C., BELBASE, K., BLACK, M., CHUMLES, W. C., COLE, T., FRONGILLO, E., GRUMMER-STRAWN, L., MARTORELL, R., SHRIMPTON, R., VAN DEN BROECK, J., ARAUJO, C. L., VICTORA, C. G., ALBERNAZ, E., TOMASI, E., DA SILVEIRA, R. D. F., NADER, G., LARTEY, A., OWUSU, W. B., SAGOE-MOSES, I., GOMEZ, V., SAGOE-MOSES, C.,

BHANDARI, N., BHAN, M. K., TANEJA, S., RONGSEN, T., CHETIA, J., SHARMA, P., BAHL, R., BJOERNEBOE, G. E. A., BAERUG, A., TUFTE, E., NORUM, K. R., RUDVIN, K., NYSAETHER, H., MOHAMED, A. J., ALASFOOR, D., PRAKASH, N. S., MABRY, R. M., AL RAJAB, H. J., HELMI, S. A., DEWEY, K. G., NOMMSEN-RIVERS, L. A., COHEN, R. J., HEINIG, M. J. & S, W. M. G. R. 2006. Enrolment and baseline characteristics in the WHO Multicentre Growth Reference Study. *Acta Paediatrica,* 95, 7-15.

OSATHANONDH, V. & POTTER, E. L. 1963a. Development of Human Kidney as Shown by Microdissection. Ii. Renal Pelvis, Calyces, and Papillae. *Arch Pathol,* 76, 277-89.

OSATHANONDH, V. & POTTER, E. L. 1963b. Development of Human Kidney as Shown by Microdissection. Iii. Formation and Interrelationship of Collecting Tubules and Nephrons. *Arch Pathol,* 76, 290-302.

OSKOUI, M., COUTINHO, F., DYKEMAN, J., JETTE, N. & PRINGSHEIM, T. 2013. An update on the prevalence of cerebral palsy: a systematic review and meta-analysis. *Developmental Medicine and Child Neurology,* 55, 509-519.

OZAKI, T., NISHINA, H., HANSON, M. A. & POSTON, L. 2001. Dietary restriction in pregnant rats causes gender-related hypertension and vascular dysfunction in offspring. *J Physiol,* 530, 141-52.

PAINTER, R. C., ROSEBOOM, T. J. & BLEKER, O. P. 2005. Prenatal exposure to the Dutch famine and disease in later life: An overview. *Reproductive Toxicology,* 20, 345-352.

PARK, J.-S. & KOPAN, R. 2016. Chapter 8 - Notch Signaling in Nephron Segmentation A2 - Little, Melissa H. *Kidney Development, Disease, Repair and Regeneration.* San Diego: Academic Press.

PEACOCK, J. L., BLAND, J. M. & ANDERSON, H. R. 1995. Preterm delivery: effects of socioeconomic factors, psychological stress, smoking, alcohol, and caffeine. *BMJ,* 311, 531-5.

PERRONE, S., MUSSAP, M., LONGINI, M., FANOS, V., BELLIENI, C. V., PROIETTI, F., CATALDI, L. & BUONOCORE, G. 2007. Oxidative kidney damage in preterm newborns during perinatal period. *Clin Biochem,* 40, 656-60.

PICONE, S. & PAOLILLO, P. 2010. Neonatal outcomes in a population of late-preterm infants. *J Matern Fetal Neonatal Med,* 23 Suppl 3, 116-20.

POEHLMANN, J., SCHWICHTENBERG, A. J., BOLT, D. & DILWORTH-BART, J. 2009. Predictors of depressive symptom trajectories in mothers of preterm or low birth weight infants. *J Fam Psychol,* 23, 690-704.

POLAK, J. F., PENCINA, M. J., PENCINA, K. M., O'DONNELL, C. J., WOLF, P. A. & D'AGOSTINO, R. B., SR. 2011. Carotid-wall intima-media thickness and cardiovascular events. *N Engl J Med,* 365, 213-21.

POTTER, E. 1965. Development of the human glomerulus. *Arch Pathol,* 8, 2917-2923. POWELL, H., MCCAFFERY, K., MURPHY, V. E., HENSLEY, M. J., CLIFTON, V. L., GILES, W. & GIBSON, P. G. 2013. Psychosocial variables are related to future exacerbation risk and perinatal outcomes in pregnant women with asthma. *J Asthma,* 50, 383-9.

PULVER, L. S., GUEST-WARNICK, G., STODDARD, G. J., BYINGTON, C. L. & YOUNG, P. C. 2009. Weight for gestational age affects the mortality of late preterm infants. *Pediatrics,* 123, e1072-7.

RABI, Y., SINGHAL, N. & NETTEL-AGUIRRE, A. 2011. Room-air versus oxygen administration for resuscitation of preterm infants: the ROAR study. *Pediatrics,* 128, e374-81.

RAY, J. G., VERMEULEN, M. J., SHAPIRO, J. L. & KENSHOLE, A. B. 2001. Maternal and neonatal outcomes in pregestational and gestational diabetes mellitus, and the influence of maternal obesity and weight gain: the DEPOSIT study. Diabetes Endocrine Pregnancy Outcome Study in Toronto. *QJM,* 94, 347-56.

REGINENSI, A., CLARKSON, M., NEIRIJNCK, Y., LU, B., OHYAMA, T., GROVES, A. K., SOCK, E., WEGNER, M., COSTANTINI, F., CHABOISSIER, M. C. & SCHEDL, A. 2011. SOX9 controls epithelial branching by activating RET effector genes during kidney development. *Hum Mol Genet,* 20, 1143-53.

ROBERT, B., ST JOHN, P. L., HYINK, D. P. & ABRAHAMSON, D. R. 1996. Evidence that embryonic kidney cells expressing flk-1 are intrinsic, vasculogenic angioblasts. *Am J Physiol,* 271, F744-53.

ROBERTS, D. & DALZIEL, S. 2006. Antenatal corticosteroids for accelerating fetal lung maturation for women at risk of preterm birth. *Cochrane Database Syst Rev,* 3, CD004454.

ROBERTS, G., CHEONG, J., OPIE, G., CARSE, E., DAVIS, N., DUFF, J., LEE, K. J., DOYLE, L. & VICTORIAN INFANT COLLABORATIVE STUDY, G. 2013. Growth of extremely preterm

survivors from birth to 18 years of age compared with term controls. *Pediatrics,* 131, e439-45.

ROBILLARD, J. E., RAMBERG, E., SESSIONS, C., CONSAMUS, B., VAN ORDEN, D., WEISMANN, D. & SMITH, F. G., JR. 1980. Role of aldosterone on renal sodium and potassium excretion during fetal life and newborn period. *Dev Pharmacol Ther,* 1, 201-16.

RODESCH, F., SIMON, P., DONNER, C. & JAUNIAUX, E. 1992. Oxygen measurements in endometrial and trophoblastic tissues during early pregnancy. *Obstet Gynecol,* 80, 283-5.

RODRIGUEZ-SORIANO, J., AGUIRRE, M., OLIVEROS, R. & VALLO, A. 2005. Long-term renal follow-up of extremely low birth weight infants. *Pediatr Nephrol,* 20, 579-84.

RODRIGUEZ, M. M., GOMEZ, A., ABITBOL, C., CHANDAR, J., MONTANE, B. & ZILLERUELO, G. 2005. Comparative renal histomorphometry: a case study of oligonephropathy of prematurity. *Pediatr Nephrol,* 20, 945-9.

RODRIGUEZ, M. M., GOMEZ, A. H., ABITBOL, C. L., CHANDAR, J. J., DUARA, S. & ZILLERUELO, G. E. 2004. Histomorphometric analysis of postnatal glomerulogenesis in extremely preterm infants. *Pediatr Dev Pathol,* 7, 17-25.

ROMAGNANI, P. 2009. Toward the Identification of a "Renopoietic System"? *Stem Cells,* 27, 2247-2253.

ROSENBERG, T. J., GARBERS, S., LIPKIND, H. & CHIASSON, M. A. 2005. Maternal obesity and diabetes as risk factors for adverse pregnancy outcomes: differences among 4 racial/ethnic groups. *Am J Public Health,* 95, 1545-51.

ROSS, B., COWETT, R. & OH, W. 1977. Renal functions of low birth weight infants during the first two months of life. *Pediatr Res,* 11, 1162-1164.

RUBIN, M. I., BRUCK, E., RAPOPORT, M., SNIVELY, M., MCKAY, H. & BAUMLER, A. 1949. Maturation of Renal Function in Childhood: Clearance Studies. *J Clin Invest,* 28, 1144-62.

RUDOLPH, A. M., HEYMANN, M. A., TERAMO, K. A. W., BARRETT, C. T. & RAIHA, N. C. R. 1971. Studies on the circulation of the previable human fetus. *Pediatric Research,* 5, 452- 465.

SATLIN, L. M., WODA, C. B. & SCHWARTZ, G. J. 2003. Development of function in the metanephric kidney. *In:* VIZE, P. D., WOOLF, A. & BARD, J. B. L. (eds.) *The Kidney: From Normal Development to Congenital Disease.* Sydney: Academic Press.

SAUGSTAD, O. D. 2001. Update on oxygen radical disease in neonatology. *Curr Opin Obstet Gynecol,* 13, 147-53.

SAXEN, L. & SARIOLA, H. 1987. Early organogenesis of the kidney. *Pediatr Nephrol,* 1, 385-92.

SCHIEVE, L. A. & HANDLER, A. 1996. Preterm delivery and perinatal death among black and white infants in a Chicago-area perinatal registry. *Obstet Gynecol,* 88, 356-63.

SCHLONDORFF, D. 1987. The glomerular mesangial cell: an expanding role for a specialized pericyte. *FASEB J,* 1, 272-81.

SCHNEIDER, D. J. & MOORE, J. W. 2006. Patent ductus arteriosus. *Circulation,* 114, 1873-82.

SCHOLL, T. O., SALMON, R. W., MILLER, L. K., VASILENKO, P., 3RD, FUREY, C. H. & CHRISTINE, M. 1988. Weight gain during adolescent pregnancy. Associated maternal characteristics and effects on birth weight. *J Adolesc Health Care,* 9, 286-90.

SCHREUDER, M., DELEMARRE-VAN DE WAAL, H. & VAN WIJK, A. 2006. Consequences of Intrauterine Growth Restriction for the Kidney. *Kidney and Blood Pressure Research,* 29, 108-125.

SCHREUDER, M. F., WILHELM, A. J., BOKENKAMP, A., TIMMERMANS, S. M., DELEMARRE-VAN DE WAAL, H. A. & VAN WIJK, J. A. 2009. Impact of gestational age and birth weight on amikacin clearance on day 1 of life. *Clin J Am Soc Nephrol,* 4, 1774-8.

SEELY, E. W. & MAXWELL, C. 2007. Cardiology patient page. Chronic hypertension in pregnancy. *Circulation,* 115, e188-90.

SHAH, P. S., ZAO, J., AL-WASSIA, H., SHAH, V. & KNOWLEDGE SYNTHESIS GROUP ON DETERMINANTS OF PRETERM, L. B. W. B. 2011. Pregnancy and neonatal outcomes of aboriginal women: a systematic review and meta-analysis. *Womens Health Issues,* 21, 28-39.

SIBAI, B., DEKKER, G. & KUPFERMINC, M. 2005. Pre-eclampsia. *Lancet,* 365, 785-799.

SIBAI, B. M. 2003. Diagnosis and management of gestational hypertension and preeclampsia. *Obstet Gynecol,* 102, 181-92.

SIEGEL, S. R. & OH, W. 1976. Renal function as a marker of human fetal maturation. *Acta Paediatr Scand,* 65, 481-5.

SIEWERT-DELLE, A. & LJUNGMAN, S. 1998. The impact of birth weight and gestational age on blood pressure in adult life. A population-based study of 49-year-old men. *AJH,* 11, 946-953.

SIMON, M., GRONE, H. J., JOHREN, O., KULLMER, J., PLATE, K. H., RISAU, W. & FUCHS, E. 1995. Expression of vascular endothelial growth factor and its receptors in human renal ontogenesis and in adult kidney. *Am J Physiol,* 268, F240-50.

SMITH, A. P., ONG, S., SMITH, N. C. & CAMPBELL, D. 2001. A prospective longitudinal study of growth velocity in twin pregnancy. *Ultrasound Obstet Gynecol,* 18, 485-7.

SMITH, L. E. 2003. Pathogenesis of retinopathy of prematurity. *Semin Neonatol,* 8, 469-73.

SMITH, L. J., MCKAY, K. O., VAN ASPEREN, P. P., SELVADURAI, H. & FITZGERALD, D. A. 2010. Normal development of the lung and premature birth. *Paediatr Respir Rev,* 11, 135-42.

SOKOL, R. J., JANISSE, J. J., LOUIS, J. M., BAILEY, B. N., AGER, J., JACOBSON, S. W. & JACOBSON, J. L. 2007. Extreme prematurity: an alcohol-related birth effect. *Alcohol Clin Exp Res,* 31, 1031-7.

STAPLETON, F., JONES, D. & GREEN, R. 1987. Acute renal failure in neonates: Incidence, etiology and outcome. *Pediatr Nephrol,* 1, 314-320.

STEVENS, L. A., CORESH, J., GREENE, T. & LEVEY, A. S. 2006. Assessing kidney function--measured and estimated glomerular filtration rate. *N Engl J Med,* 354, 2473-83.

STEWART, J. H., MCCREDIE, M. R. & MCDONALD, S. P. 2004. The incidence of treated end- stage renal disease in New Zealand Maori and Pacific Island people and in Indigenous Australians. *Nephrol Dial Transplant,* 19, 678-85.

STOLL, B. J., HANSEN, N. I., BELL, E. F., SHANKARAN, S., LAPTOOK, A. R., WALSH, M. C., HALE, E. C., NEWMAN, N. S., SCHIBLER, K., CARLO, W. A., KENNEDY, K. A., POINDEXTER, B. B., FINER, N. N., EHRENKRANZ, R. A., DUARA, S., SANCHEZ, P. J., O'SHEA, T. M., GOLDBERG, R. N., VAN MEURS, K. P., FAIX, R. G., PHELPS, D. L., FRANTZ, I. D., 3RD, WATTERBERG, K. L., SAHA, S., DAS, A., HIGGINS, R. D., EUNICE KENNEDY SHRIVER NATIONAL INSTITUTE OF CHILD, H. & HUMAN DEVELOPMENT NEONATAL RESEARCH,
N. 2010. Neonatal outcomes of extremely preterm infants from the NICHD Neonatal Research Network. *Pediatrics,* 126, 443-56.

SULYOK, E., VARG, F., GYORY, E., JOBST, K. & CZABA, I. 1979. Postnatal development of renal sodium handling in premature infants. *J Pediatr,* 95, 787-792.

SUTHERLAND, M. R., GUBHAJU, L., MOORE, L., KENT, A. L., DAHLSTROM, J. E., HORNE, R. S., HOY, W. E., BERTRAM, J. F. & BLACK, M. J. 2011. Accelerated maturation and abnormal morphology in the preterm neonatal kidney. *J Am Soc Nephrol,* 22, 1365-74.

SUTHERLAND, M. R., GUBHAJU, L., YODER, B. A., STAHLMAN, M. T. & BLACK, M. J. 2009. The effects of postnatal retinoic acid administration on nephron endowment in the preterm baboon kidney. *Pediatr Res,* 65, 397-402.

SWEENEY, W. & AVNER, E. 2004. The kidney - Embryogenesis and anatomic development of the kidney. *In:* POLIN, R., FOX, W. & ABMAN, S. (eds.) *Fetal and Nenatal Physiology.* 3 ed.: Saunders (Elsevier).

TAAL, M. W. & BRENNER, B. M. 2007. Defining renal risk. *Curr Opin Nephrol Hypertens,* 16, 554-6.

TAKIEDDINE, F., TABBARA, M., HALL, P., SOKOL, R. J. & KING, K. C. 1983. Fetal renal maturation. Studies on urinary beta 2 microglobulin the neonate. *Acta Obstet Gynecol Scand,* 62, 311-4.

TAN, A., SCHULZE, A., O'DONNELL, C. P. & DAVIS, P. G. 2005. Air versus oxygen for resuscitation of infants at birth. *Cochrane Database Syst Rev*, CD002273.

TANG, J., ZHU, Z., XIA, S., LI, N., CHEN, N., GAO, Q., LI, L., ZHOU, X., LI, D., ZHU, X., TU, Q., LI, W., WU, C., LI, J., ZHONG, Y., LI, X., MAO, C. & XU, Z. 2015. Chronic hypoxia in pregnancy affected vascular tone of renal interlobar arteries in the offspring. *Sci Rep,* 5, 9723.

TARVER-CARR, M. E., POWE, N. R., EBERHARDT, M. S., LAVEIST, T. A., KINGTON, R. S., CORESH, J. & BRANCATI, F. L. 2002. Excess risk of chronic kidney disease among African-American versus white subjects in the United States: a population-based study of potential explanatory factors. *J Am Soc Nephrol,* 13, 2363-70.

THAYYIL, S., SHEIK, S., KEMPLEY, S. T. & SINHA, A. 2008. A gestation- and postnatal age-based reference chart for assessing renal function in extremely premature infants. *J Perinatol,* 28, 226-9.

THOMAS, G., SEHGAL, A. R., KASHYAP, S. R., SRINIVAS, T. R., KIRWAN, J. P. & NAVANEETHAN, S. D. 2011. Metabolic syndrome and kidney disease: a systematic review and meta-analysis. *Clin J Am Soc Nephrol,* 6, 2364-73.

THORNTON, J. 2013. Initiation of aspirin in early gestation for the prevention of pre- eclampsia. *Bjog-an International Journal of Obstetrics and Gynaecology,* 120, 774-775.

TOMLINSON, P. A. 1992. Low molecular weight proteins in children with renal disease. *Pediatric Nephrology,* 6, 565-71.

TORLONI, M. R., BETRAN, A. P., HORTA, B. L., NAKAMURA, M. U., ATALLAH, A. N., MORON, A. F. & VALENTE, O. 2009. Prepregnancy BMI and the risk of gestational diabetes: a systematic review of the literature with meta-analysis. *Obes Rev,* 10, 194-203.

TSUKAHARA, H., FUJII, Y., TSUCHIDA, S., HIRAOKA, M., MORIKAWA, K., HARUKI, S. & SUDO, M. 1994. Renal handling of albumin and beta-2-microglobulin in neonates. *Nephron,* 68, 212-6.

TSUKAHARA, H., YOSHIMOTO, M., SAITO, M., SAKAGUCHI, T., MITSUYOSHI, I., HAYASHI, S., NAKAMURA, K., KIKUCHI, K. & SUDO, M. 1990. Assessment of tubular function in neonates using urinary beta 2-microglobulin. *Pediatric Nephrology,* 4, 512-4.

TUCKER, J. & MCGUIRE, W. 2004. Epidemiology of preterm birth. *BMJ,* 329, 675-8.

TUFRO-MCREDDIE, A., NORWOOD, V. F., AYLOR, K. W., BOTKIN, S. J., CAREY, R. M. & GOMEZ, R. A. 1997. Oxygen regulates vascular endothelial growth factor-mediated vasculogenesis and tubulogenesis. *Dev Biol,* 183, 139-49.

VAN MARTER, L. J., DAMMANN, O., ALLRED, E. N., LEVITON, A., PAGANO, M., MOORE, M., MARTIN, C. & DEVELOPMENTAL EPIDEMIOLOGY NETWORK, I. 2002. Chorioamnionitis, mechanical ventilation, and postnatal sepsis as modulators of chronic lung disease in preterm infants. *J Pediatr,* 140, 171-6.

VANGEN, S., STOLTENBERG, C., HOLAN, S., MOE, N., MAGNUS, P., HARRIS, J. R. & STRAY-PEDERSEN, B. 2003. Outcome of pregnancy among immigrant women with diabetes. *Diabetes Care,* 26, 327-32.

VENTO, M., ASENSI, M., SASTRE, J., LLORET, A., GARCIA-SALA, F. & VINA, J. 2003. Oxidative stress in asphyxiated term infants resuscitated with 100% oxygen. *J Pediatr,* 142, 240-6.

VENTURA, S. J., MARTIN, J. A., CURTIN, S. C. & MATHEWS, T. J. 1998. Report of final natality statistics, 1996. *Mon Vital Stat Rep,* 46, 1-99.

VIKSE, B. E., IRGENS, L. M., LEIVESTAD, T., HALLAN, S. & IVERSEN, B. M. 2008. Low birth weight increases risk for end-stage renal disease. *J Am Soc Nephrol,* 19, 151-7.

VILLAR, J., GIULIANI, F., BHUTTA, Z. A., BERTINO, E., OHUMA, E. O., ISMAIL, L. C., BARROS, F. C., ALTMAN, D. G., VICTORA, C., NOBLE, J. A., GRAVETT, M. G., PURWAR, M., PANG, R., LAMBERT, A., PAPAGEORGHIOU, A. T., OCHIENG, R., JAFFER, Y. A., KENNEDY, S. H., INTERNATIONAL, F. & NEWBORN GROWTH CONSORTIUM FOR THE, C. 2015. Postnatal growth standards for preterm infants: the Preterm Postnatal Follow-up Study of the INTERGROWTH-21(st) Project. *Lancet Glob Health,* 3, e681-91.

VINCER, M. J., ALLEN, A. C., JOSEPH, K. S., STINSON, D. A., SCOTT, H. & WOOD, E. 2006. Increasing prevalence of cerebral palsy among very preterm infants: A population-based study. *Pediatrics,* 118, E1621-E1626.

VOLPE, J. J. 2008. Postnatal sepsis, necrotizing entercolitis, and the critical role of systemic inflammation in white matter injury in premature infants. *Journal of Pediatrics,* 153, 160-163.

WALKER, B. R., MCCONNACHIE, A., NOON, J. P., WEBB, D. J. & WATT, G. C. 1998. Contribution of parental blood pressures to association between low birth weight and adult high blood pressure: cross sectional study. *BMJ,* 316, 834-7.

WALKER, M. W., CLARK, R. H. & SPITZER, A. R. 2011. Elevation in plasma creatinine and renal failure in premature neonates without major anomalies: terminology, occurrence and factors associated with increased risk. *J Perinatol,* 31, 199-205.

WANG, Y., CHEN, X., SONG, Y., CABALLERO, B. & CHESKIN, L. J. 2008. Association between obesity and kidney disease: a systematic review and meta-analysis. *Kidney Int,* 73, 19-33.

WEN, C. P., MATSUSHITA, K., CORESH, J., ISEKI, K., ISLAM, M., KATZ, R., MCCLELLAN, W., PERALTA, C. A., WANG, H., DE ZEEUW, D., ASTOR, B. C., GANSEVOORT, R. T., LEVEY, A. S., LEVIN, A. & CHRONIC KIDNEY DISEASE PROGNOSIS, C. 2014. Relative risks of chronic kidney disease for mortality and end-stage renal disease across races are similar. *Kidney Int,* 86, 819-27.

WHARRAM, B. L., GOYAL, M., WIGGINS, J. E., SANDEN, S. K., HUSSAIN, S., FILIPIAK, W. E., SAUNDERS, T. L., DYSKO, R. C., KOHNO, K., HOLZMAN, L. B. & WIGGINS, R. C. 2005. Podocyte depletion causes glomerulosclerosis: diphtheria toxin-induced podocyte depletion in rats expressing human diphtheria toxin receptor transgene. *J Am Soc Nephrol,* 16, 2941-52.

WHITE, S. L., PERKOVIC, V., CASS, A., CHANG, C. L., POULTER, N. R., SPECTOR, T., HAYSOM, L., CRAIG, J. C., SALMI, I. A., CHADBAN, S. J. & HUXLEY, R. R. 2009. Is low birth weight an antecedent of CKD in later life? A systematic review of observational studies. *Am J Kidney Dis,* 54, 248-61.

WILKINS, B. H. 1992. Renal function in sick very low birthweight infants: 4. Glucose excretion. *Arch Dis Child,* 67, 1162-5.

WINTERBOURN, C. C. 2008. Reconciling the chemistry and biology of reactive oxygen species. *Nat Chem Biol,* 4, 278-86.

WOLLMANN, H. A. 1998. Intrauterine growth restriction: definition and etiology. *Horm Res,* 49, 1-6.

WOOLF, A. S., WINYARD, P. J., HERMANNS, M. H. & WELHAM, S. M. J. 2003. Maldevelopment of the Human Kidney and Lower Urinary Tract: An Overview. *In:* VIZE PD, W. A., BARD JBL, (ed.) *The Kidney: From Normal Development to Congenital Disease.* Academic Press.

YANG, B., CROSS, D. F., OLLERENSHAW, M., MILLWARD, B. A. & DEMAINE, A. G. 2003. Polymorphisms of the vascular endothelial growth factor and susceptibility to diabetic microvascular complications in patients with type 1 diabetes mellitus. *J Diabetes Complications,* 17, 1-6.

YOSYPIV, I. V. 2008. A new role for the renin-angiotensin system in the development of the ureteric bud and renal collecting system. *Keio J Med,* 57, 184-9.

YZYDORCZYK, C., COMTE, B., CAMBONIE, G., LAVOIE, J. C., GERMAIN, N., TING SHUN, Y., WOLFF, J., DESCHEPPER, C., TOUYZ, R. M., LELIEVRE-PEGORIER, M. & NUYT, A. M. 2008. Neonatal oxygen exposure in rats leads to cardiovascular and renal alterations in adulthood. *Hypertension,* 52, 889-95.

ZHUO, J. L. & LI, X. C. 2013. Proximal nephron. *Compr Physiol,* 3, 1079-123.

ZIMANYI, M. A., DENTON, K. M., FORBES, J. M., THALLAS-BONKE, V., THOMAS, M. C., POON, F. & BLACK, M. J. 2006. A developmental nephron deficit in rats is associated with increased susceptibility to a secondary renal injury due to advanced glycation end-products. *Diabetologia,* 49, 801-10.

ZOHDI, V., MORITZ, K. M., BUBB, K. J., COCK, M. L., WREFORD, N., HARDING, R. & BLACK, M. J. 2007. Nephrogenesis and the renal renin-angiotensin system in fetal sheep: effects of intrauterine growth restriction during late gestation. *Am J Physiol Regul Integr Comp Physiol,* 293, R1267-73.

REVIEW 1:
LONG-TERM RENAL CONSEQUENCES OF PRETERM BIRTH

CHAPTER ONE (REVIEW 1) DECLARATION

Declaration by candidate
[It is to be noted that I have used my married surname (Ryan) in this publication]

Chapter 1- Review 1 was published in Clinical Perinatology in 2014. Reprinted in this thesis is a copy of the final published manuscript. Sutherland (2014). "Long-Term Renal Consequences of Preterm Birth". Clin Perinatol 41 (2). 561 – 573.

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

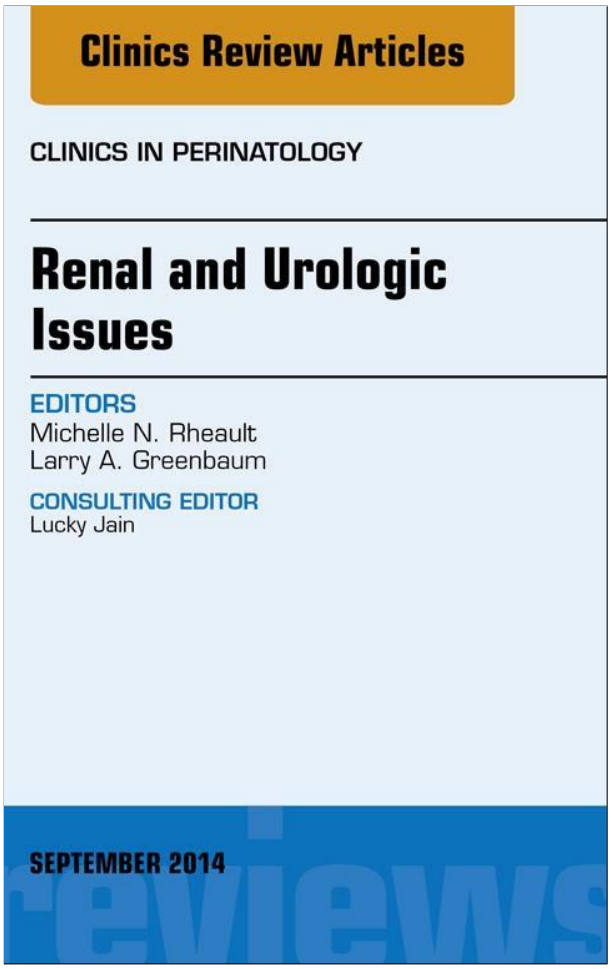
Nature of contribution	Extent of contribution (%)
Co-authored manuscript	35 %

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

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Megan Sutherland, Alison Kent, M. Jane Black	Assisted with writing of manuscript	N

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

Candidate's Signature	[redacted]	Date 21.9.16
Main Supervisor's Signature	[redacted]	Date 21.9.16



Long-Term Renal Consequences of Preterm Birth

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KEYWORDS

• Glomeruli • Preterm • Chorioamnionitis • Diabetes • Preeclampsia
• Growth restriction • Antenatal steroids

KEY POINTS

- Several antenatal factors have the potential to impair kidney development, including fetal growth restriction, maternal hypertension, and diabetes.
- Preterm birth is associated with several postnatal risk factors for kidney development, including increased physiologic requirements related to ex utero life, nephrotoxic medications, acute kidney injury, and postnatal growth failure.
- Children and adults born preterm may have reduced kidney size and increased blood pressure (BP), which likely predispose to renal disease later in life.
- The population of individuals born preterm continues to increase worldwide; it is expected that further evidence of renal dysfunction after preterm birth will continue to emerge in the future.

CLINICAL SCENARIO 1

A woman presented to the delivery suite at 2611 weeks' gestation with ruptured membranes and was managed with intravenous ampicillin 500 mg 6 hourly, gentamicin 120 mg daily for 48 hours and then converted to oral azithromycin 500 mg every three days. She received antenatal steroids (betamethasone 12 mg daily for 2 doses) and was admitted to the antenatal ward. Four days later, she developed a fever and tachycardia, with an associated increase in white cell count on full blood picture and increased C-reactive protein levels. Labor ensued, and she delivered a

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2616 week gestation male infant weighing 900 g (50th centile), who required intubation for resuscitation, received a dose of surfactant, and was extubated at 48 hours of age. Placental histology later confirmed the diagnosis of chorioamnionitis and funisitis. Certain components of this history have long-term implications on renal health (Fig. 1).

Chorioamnionitis

Chorioamnionitis (bacterial infection that causes inflammation of the amnion and chorion), is widely recognized as a significant contributor to preterm birth, as a cause of both spontaneous preterm labor and premature rupture of the amniotic membrane.[1,2] Chorioamnionitis also produces the fetal inflammatory response syndrome (FIRS), characterized by an inflamed umbilical cord and increased fetal serum levels of proinflammatory cytokines.[3] Consequently, FIRS can adversely influence neonatal organ development, including the lungs,[4] brain,[2,5] thymus,[6,7] and kidney.[8] In an ovine model, intrauterine exposure to chorioamnionitis (bolus intra-amniotic high dose of endotoxin [10 mg lipopolysaccharide (LPS)] at a time in gestation when nephrogenesis was near completion) was found to cause a 20% reduction in nephron endowment.[8] In response to the reduction in nephron number, there was also a significant increase in glomerular volume, most likely resulting from compensatory hypertrophy; these adverse effects on nephrogenesis occurred in the absence of fetal growth restriction.[8] This finding is clinically important, because a reduction in nephron number can lead to increased susceptibility to renal injury and disease in later life.[9–14] In a more recent ovine study,[15] it was found that exposure to a chronic low-dose intra-amniotic infusion of endotoxin (1 mg LPS), during a time when fetal nephrogenesis was still rapidly ongoing, did not lead to any observable deleterious effects on nephron endowment. Together, these findings are indicative that the extent of infection as well as the timing (acute vs chronic) may influence the impact of chorioamnionitis on renal development.

Antenatal Steroids

Besides the beneficial effects on lung function and postnatal survival, antenatal glucocorticoid treatment is also associated with increased mean arterial BP, and increased

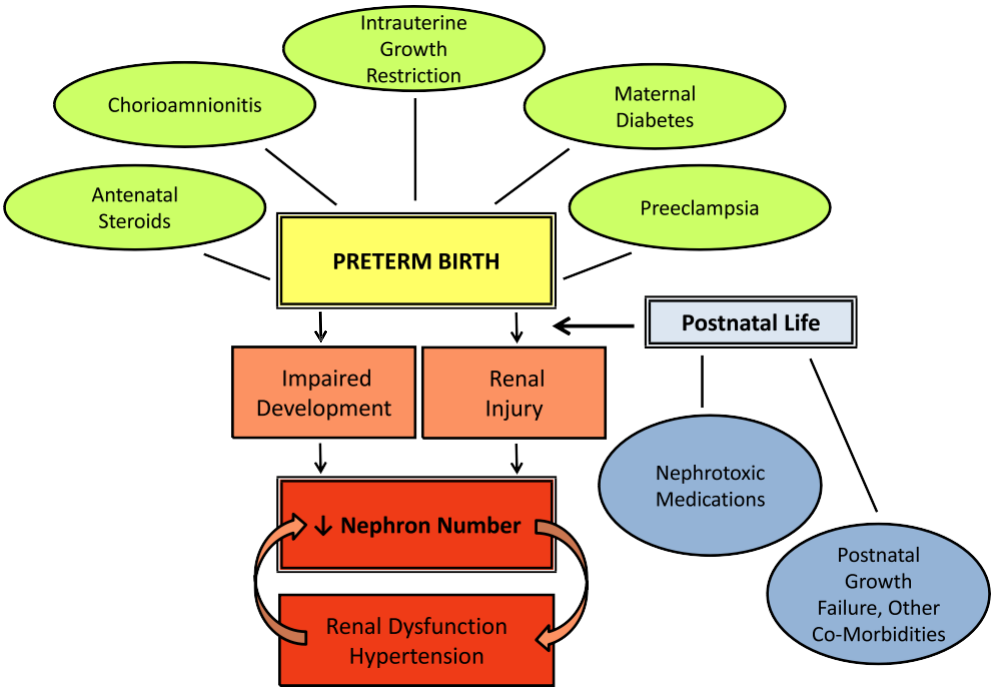

Fig. 1. Prenatal and postnatal risk factors for renal developmental injury.

renal blood flow and glomerular filtration rate (GFR),[16] indicating that accelerated functional maturation of the kidney also occurs after treatment. Similarly, in studies conducted in a baboon model of preterm birth,[17] there was evidence of accelerated renal maturation with a greater number of mature nephrons present in the glucocorticoid-exposed kidneys when examined both at the time of preterm delivery and at postnatal day 21. Although it has not been established, it is possible that this accelerated maturation results in an earlier cessation of nephrogenesis. In this regard, in other studies performed in animal models (including rodents[18–20] and sheep[21]), antenatal glucocorticoid administration was shown to lead to a significant reduction in nephron endowment of the exposed offspring. A study by de Vries and colleagues[22] 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.

Preterm Birth

The transition from an intrauterine to an extrauterine environment involves a sudden increase in blood oxygen concentrations, as well as increased systemic BP and organ perfusion. The effects that this hemodynamic shift may have on the immature developing kidney (with nephrogenesis ongoing at the time of preterm birth) are poorly understood. After birth, the preterm kidney is functionally immature, with a low GFR, inability to maintain electrolyte balance,[23] and proteinuria.[24] Acute kidney injury is also common.[25] Some (but not all[26]) studies of kidney size in preterm neonates have shown significantly reduced kidney length or volume compared with neonates born at term.[27–30]

Histomorphologic studies[31] of renal tissue collected at autopsy have shown accelerated postnatal maturation of the preterm kidney, with reduced nephrogenic zone width, reduced renal vesicle formation, and increased number of glomerular generations compared with age-matched fetal controls. At the completion of nephrogenesis, a reduction in the number of glomerular generations formed (suggestive of a nephron deficit) has also been reported.[10] In addition, glomeruli that are abnormal in appearance (enlarged Bowman space and shrunken glomerular tuft) were present in the outer renal cortex of preterm kidneys[31]; in some neonates, up to 13% of glomeruli were affected. These glomeruli have scant capillarization, suggestive of impaired vascular development or injury, which is likely to render them nonfunctional.[17,32] In a baboon model of preterm birth, nephron density was found to be significantly lower in the preterm kidneys, and nephron number was at the lower end of the normal distribution,[17] and in mice delivered 1 to 2 days preterm, nephron number was significantly reduced.[33] Together, these findings suggest that preterm neonates may have both a reduced endowment of nephrons at the completion of nephrogenesis, as well a predisposition to nephron loss in the neonatal period, which may have important implications for long-term renal health.

CLINICAL SCENARIO 1 (CONTINUED)

Because of the maternal history of clinical chorioamnionitis, the preterm infant was commenced on ampicillin 25 mg/kg/dose 12 hourly and gentamicin 2.5 mg/kg/dose 24 hourly, which were ceased at 48 hours of age after a negative blood culture. On day 2 of life, an echocardiogram was performed, and the infant was found to have a large patent ductus arteriosus, with an increased left atrium/aorta ratio. He was commenced on intravenous indomethacin 0.2 mg/kg first dose, 0.1 mg/kg subsequent doses daily for 5 days; however, at the end of the third day of treatment, he developed abdominal distension, bilious aspirates, and hypotension and had an abdominal radiograph, with signs consistent with necrotizing enterocolitis. He

required reintubation and inotropes, was not fed enterally for 10 days, and received 7 days of ampicillin 25 mg/kg/dose 12 hourly, gentamicin 2.5 mg/kg/dose 24 hourly, and metronidazole 7.5 mg/kg/dose 48 hourly. He was recommenced on feeds but continued to have significant feed intolerance until full feeds were achieved on day 30. He was extubated at 28 days of age, requiring dexamethasone, and continued to require continuous positive airway pressure (CPAP) until 35 weeks postconceptional age and oxygen until 38 weeks postconceptional age. His weight on discharge was 2.2 kg, which was less than the 3rd centile, having decreased from the 50th centile at birth. This relatively common series of complications of extreme prematurity have further renal implications.

Nephrotoxic Medications

The prenatal and postnatal administration of a variety of nephrotoxic medications is common practice in the setting of preterm birth.[34–38] Experimental studies have shown that antenatal exposure to the most commonly administered aminoglycoside antibiotic, gentamicin, causes significant reductions in nephron endowment.[39] Gentamicin accumulates in renal proximal tubule cells, leading to cellular necrosis[40]; postnatally, this results in increased sodium excretion, proteinuria, and reduced GFR in affected neonates.[41–43] Exposure of the developing kidney to b-lactam antibiotics (such as ampicillin) has also been shown to result in impaired nephrogenesis and cystic tubular dilation.[44]

The consequences of antenatal nonsteroidal antiinflammatory drugs (NSAIDs) (indomethacin, ibuprofen) exposure on the fetal kidney is well described both in experimental models (nephron deficits, reduced cortical renal volume,[45–47] and severe renal dysplasia[48]) and in human neonates (glomerular cysts and renal dysfunction[49,50]). However, less is known about the effects of postnatal NSAID exposure on the kidney. In general, the administration of NSAIDs causes systemic vasoconstriction, which in turn leads to significant reductions in renal blood flow and urine output.[51–53] A study in human preterm neonates[54] recently showed significantly increased number of podocytes in the urine, and increased urine albumin excretion in those treated with indomethacin after birth, which indicates glomerular injury. Furthermore, an experimental study conducted in a neonatal rodent model[55] showed that postnatal administration of NSAIDs or gentamicin led to proximal tubule vacuolization, interstitial edema, and podocyte foot process effacement; the most severe effects were observed in animals that received combined NSAID and gentamicin treatment. In a nonhuman primate (baboon) model,[56] animals administered ibuprofen after preterm birth had a significantly reduced nephrogenic zone width. This finding suggests that prostaglandin inhibition may impair nephrogenesis. Kent and colleagues[57] in their early study reported that there was no effect of early postnatal NSAID and gentamicin exposure on nephron endowment in a rat model at 14 days but have now shown that the adult rat has a significantly reduced nephron number after early administration of indomethacin but not ibuprofen (Kent AL, Koina M, Gubhaju L, et al: Indomethacin administered early in the postnatal period results in reduced glomeruli in the adult rat. Submitted for publication).

Growth Restriction

Over the past decade, it has been well established that intrauterine growth restriction (IUGR) results in a reduced nephron endowment[58–62] and is linked to subsequent renal dysfunction.[9,58,63–66] The number of nephrons formed during nephrogenesis in early life determines the lifelong functional capacity of the kidney; there have been a multitude of experimental studies (in many species) showing that the number of nephrons formed within the kidney is directly proportional to kidney size at birth but may not be

consistent in fetuses that have been growth-restricted late in gestation (when nephrogenesis is completed).[17,62,67,68] IUGR is often a comorbidity of preterm birth; it is linked to the cause of spontaneous preterm birth, and is also a common cause of interventional preterm delivery, which is required to facilitate infant survival.[69] Of greatest concern, newborns who are born both prematurely and IUGR are at a high risk of infant morbidity and mortality.[70–73] Long-term studies in children and adults have found that individuals born both preterm and IUGR have an increased propensity for impaired kidney growth[28,59,74] and renal dysfunction,[75,76] compared with preterm individuals who were non-IUGR at birth. Besides impaired growth in utero, extrauterine growth restriction is a common sequela of preterm birth and has been shown to have a similarly adverse effect on renal function.[76]

CLINICAL SCENARIO 2

A woman presented to the antenatal clinic at 2610 weeks' gestation and was found to have preeclampsia with BP of 140/95, an increased urate level, but at this stage, normal renal function and normal umbilical arterial Doppler results. She had a body mass index (calculated as weight in kilograms divided by the square of height in meters) of 40 and had insulin-requiring gestational diabetes. The estimated fetal weight was 660 g, which is on the 5th centile, with the fetal size at her 20-week morphology scan being on the 78th centile. She was admitted to the antenatal ward and commenced on oral antihypertensives (Labetalol 200 mg 8 hourly) and received antenatal steroids. Over the next 2 weeks, her BP became more difficult to control and she was on 2 antihypertensive medications on maximal doses (Labetalol, Methyldopa 250 mg 6 hourly). Her renal function began to deteriorate, and the umbilical arterial Doppler results were increased. At 2814 weeks, she was given magnesium sulfate for fetal neuroprotection, and the female neonate was delivered by cesarean section with a birth weight of 720 g (10th centile). The placental histology confirmed uteroplacental insufficiency, with accelerated maturation, increased syncytial knot formation, and areas of infarction and chorangiosis.

Maternal Disease

Diabetes

This clinical scenario has become increasingly more frequent in infants born to mothers with maternal diseases.[77,78] With the increasing prevalence of obesity and associated type 2 diabetes in the United States and other developed countries, the incidence of maternal diabetes is increasing.[79] Intrauterine exposure to maternal diabetes can lead to either macrosomia or microsomia of the fetus.[80] Fetal macrosomia, specifically asymmetric macrosomia, results in exaggerated fetal growth in response to the increased supply of glucose and other nutrients across the placenta.[81] However, conversely, in the case of severe maternal diabetes, maternal complications such as vasculopathy and nephropathy may occur, which subsequently lead to IUGR of the developing fetus.

In addition, maternal diabetes has been shown to have other adverse effects on embryogenesis, which may further increase the risk of IUGR[82] and consequently increase the susceptibility of impaired nephrogenesis and renal injury in the neonatal period. For example, in a study of Pima Indians with type 2 diabetes,[83] there was found to be a strong association with renal dysfunction after birth; 58% of infants who were exposed to diabetes in utero showed 4 times higher urinary albumin excretion (albumin/creatinine ratio >30 mg/g), compared with unexposed infants. Other studies[84] have clearly shown a risk of renal malformations in infants born to diabetic mothers;

the estimated risk of delivering a child with renal agenesis/dysgenesis is more than 3 times greater for mothers with diabetes compared with nondiabetic mothers. Animal studies have further highlighted the adverse effects of intrauterine exposure to maternal diabetes on kidney development and function. In the mouse model,[82] a high-glucose intrauterine milieu led to significantly smaller body length, kidney size, and glomerular size, and a 40% reduction in nephron endowment compared with control offspring; the level of maternal hyperglycemia predicted the severity of the adverse effects on kidney size and on glomerular endowment. There was also evidence of glomerular collapse as a result of an increase in glomerular and tubular apoptotic events.[82] In follow-up studies,[85] 20-week-old mice offspring exposed to maternal diabetes were 20% lighter, with significantly increased urinary albumin excretion, glomerular hypertrophy, renal fibrosis, and an increased propensity for the development of hypertension.

Preeclampsia

Preeclampsia is known to be a major risk factor for fetal and neonatal mortality, IUGR, and preterm birth; maternal hypertension alone also increases risk, but to a lesser extent than preeclampsia.[86] Besides inducing growth restriction via impaired uteroplacental perfusion, preeclampsia also stimulates a proinflammatory, prooxidant and antiangiogenic intrauterine environment.[87] In animal models, this setting is known to lead to IUGR, decreased kidney size, hypertension, and impaired vascularization of the developing lung[88,89]; however, potential effects on the vascular development of other organs are unknown. Among children born pre- term, maternal preeclampsia is an independent risk factor for the development of hypertension.[90]

CLINICAL SCENARIO 2 (CONTINUED)

This 28-week gestation baby required CPAP for 3 weeks, took 3 weeks to achieve full feeds, and went home at 40 weeks postconceptional age formula feeding weighing 3.1 kg (10th centile). Over the next 12 months, her weight gain increased significantly, such that her weight at 12 months of age was 11.4 kg (95th centile).

Obesity After Growth Restriction

The increasing epidemic of obesity worldwide has also seen a resultant increase in chronic renal failure,[91] and there is mounting evidence of the adverse effects of obesity on renal hemodynamics and structure.[92] The preterm infant has several early renal hits, which may compromise long-term renal function, and this is further compounded later in life by insults such as the induction of obesity; this supports the multihit nature of chronic renal disease.[93] Preterm birth and the associated renal risk factors outlined so far have the potential to significantly reduce nephron number. With the increased functional demands (as seen with obesity), there is likely to be accelerated onset and increased severity of renal disease when glomerular number (functional reserve) is low as a result of growth restriction early in life. A reduced nephron endowment subsequently leads to glomerular hypertrophy, and this is likely to be greatly accentuated when the functional demands on the kidneys are increased. Furthermore, the sustained glomerular hyperfiltration may lead to glomerular dysfunction and subsequent glomerular demise.[93] Supporting this theory, Abitbol and colleagues[94] have shown that obesity and preterm birth are additive risks in the progression of kidney disease in children.

Long-Term Consequences of Preterm Birth on the Kidney

As described, there are a multitude of factors associated with preterm birth that have the potential to impair renal development in the fetus or neonate. Impaired growth and a reduced nephron endowment are strongly linked to the later development of hypertension, glomerular injury, and renal dysfunction.[95,96] In this regard, there is now an increasing body of evidence supporting the hypothesis that preterm birth has long-term consequences for health; in particular, preterm birth has been strongly linked to increased hypertension risk in both children and adults.[97] In children born preterm, most studies have reported a reduction in kidney size compared with age-matched individuals born at term.[98–100] Zaffanello and colleagues[99] further reported a significantly decreased kidney size in children born at 26 to 28 weeks' gestation, compared with those born at 30 to 31 weeks' gestation, indicating that the severity of prematurity has an important effect on kidney growth. Only 1 study has examined the effect of preterm birth on kidney size in adulthood. In this study, Keijzer-Veen and colleagues[101] found that at 20 years of age, after preterm birth at less than 32 weeks' gestation, female adults had a significantly decreased kidney length and volume (both absolute and relative) compared with individuals born at term.

However, findings from the few studies to have examined long-term renal function after preterm birth are less conclusive. Rodriguez-Soriano and colleagues[102] and Iacobelli and colleagues[103] showed that GFR was significantly reduced in preterm-born children compared with term controls, with impairments in electrolyte excretion

Table 1
Potential causes of renal impairment in preterm neonates and potential minimization strategies

	Potential Minimization Strategies
Prenatal Factors	
FGR	Use of aspirin, clexane in early pregnancy for past history of FGR in previous pregnancies to improve placentation and reduce risk of FGR
Maternal disease (hypertension, diabetes)	Close monitoring, evidence still to be determined as to ideal target ranges for BP and blood glucose levels for optimal fetal growth
Antenatal steroids	Antenatal steroids for women at high risk of preterm delivery, not to be used just in case (ie, twin pregnancy, judicious use of multiple courses)
Chorioamnionitis	None apparent at this time
Postnatal Factors	
Increased functional demand because of premature delivery	Physiologic change which must occur for ex utero life, careful fluid balance, and BP observation
Nephrotoxic drugs	Minimize use of gentamicin, vancomycin, indomethacin. Ensure staff are aware of total gentamicin days, and use other antibiotics if further septic episodes occur
Postnatal growth failure	Careful attention to nutritional requirements, ensure adequate caloric and protein content
Acute kidney injury	Careful attention to aseptic techniques with central lines and feeding regimens to minimize sepsis/necrotizing enterocolitis, which may result in acute kidney injury with early diagnosis and initiation of management strategies.

Abbreviation: FGR, fetal growth restriction.

also evident. In children examined at 6 to 8 years of age, Iacobelli and colleagues[103] also found microalbuminuria in 8.3% of the preterm children. In contrast, other studies in children[98,105] and young adults[104,106] born preterm reported no difference in renal function compared with term controls. However, survival after extremely preterm birth is a relatively recent phenomenon. Hence, it is likely that as the increasing population worldwide of survivors of very and extremely preterm birth reach middle and older age, the adverse long-term consequences of preterm birth will become increasingly evident. We need to continue to look carefully at the evidence available and try to minimize known potential adverse effects on the developing kidney (Table 1) as well as pursuing further research in this arena to prevent long-term health sequelae.

SUMMARY

The early life environment, as first postulated by Barker,[107] has the potential to influence future cardiovascular health risks. The developing kidney of the preterm infant may be affected by several in utero and neonatal insults that may influence nephrogenesis, resulting in reduced functional nephron number at the beginning of life. This reduction in nephron number may lead to vulnerability to hypertension in adulthood, increasing cardiovascular risks for myocardial infarction and stroke, as well as impaired renal function. Ongoing research is required into the potential risks to nephrogenesis, along with ways to minimize harm and maximize nephron number and function in preterm neonates to reduce the risk of long-term renal and cardiovascular sequelae.

REFERENCES

1. Mueller-Heubach E, Rubinstein DN, Schwarz SS. Histologic chorioamnionitis and preterm delivery in different patient populations. Obstet Gynecol 1990; 75(4):622–6.
2. Goldenberg RL, Hauth JC, Andrews WW. Intrauterine infection and preterm delivery. N Engl J Med 2000;342(20):1500–7.
3. Gantert M, Been JV, Gavilanes AW, et al. Chorioamnionitis: a multiorgan disease of the fetus. J Perinatol 2010;30(Suppl 1):s21–30.
4. Kallapur SJ, Jobe AH. Contribution of inflammation to lung injury and development. Arch Dis Child Fetal Neonatal Ed 2006;91:F132–5.
5. De Felice C, Toti P, Laurini RN, et al. Early neonatal brain injury in histologic chorioamnionitis. J Pediatr 2001;138(1):101–4.
6. Toti P, De Felice C, Stumpo M, et al. Acute thymic involution in fetuses and neonates with chorioamnionitis. Hum Pathol 2000;31(9):1121–8.
7. Kunzmann S, Glogger K, Been JV, et al. Thymic changes after chorioamnionitis induced by intraamniotic lipopolysaccharide in fetal sheep. Am J Obstet Gynecol 2010;190:e471–9.
8. Galinsky R, Moss TJ, Gubhaju L, et al. Effect of intra-amniotic lipopolysaccharide on nephron number in preterm fetal sheep. Am J Physiol Renal Physiol 2011;301(2):F280–5.
9. Brenner B, Garcia D, Anderson S. Glomeruli and blood pressure. Less of one, more the other? Am J Hypertens 1988;1(4 Pt 1):335–47.
10. Rodríguez MM, Gómez AH, Abitbol CL, et al. Histomorphometric analysis of postnatal glomerulogenesis in extremely preterm infants. Pediatr Dev Pathol 2004;7(1):17–25.
11. Hoy WE, Hughson MD, Bertram JF, et al. Nephron number, hypertension, renal disease, and renal failure. J Am Soc Nephrol 2005;16(9):2557–64.

12. Zandi-Nejad K, Luyckx V, Brenner B. Adult hypertension and kidney disease: the role of fetal programming. Hypertension 2006;47(3):502–8.
13. Nehiri T, Duong Van Huyen JP, Viltard M, et al. Exposure to maternal diabetes induces salt-sensitive hypertension and impairs renal function in adult rat offspring. Diabetes 2008;57(8):2167–75.
14. Gray IP, Cooper PA, Cory BJ, et al. The intrauterine environment is a strong determinant of glucose tolerance during the neonatal period, even in prematurity. J Clin Endocrinol Metab 2002;87(9):4252–6.
15. Ryan D, Atik A, De Matteo R, et al. Chronic intrauterine exposure to endotoxin does not alter fetal nephron number or glomerular size. Clin Exp Pharmacol Physiol 2013;40(11):789–94.
16. Jahnukainen T, Chen M, Berg U, et al. Antenatal glucocorticoids and renal function after birth. Semin Neonatol 2001;6(4):351–5.
17. Gubhaju L, Sutherland MR, Yoder BA, et al. Is nephrogenesis affected by preterm birth? Studies in a non-human primate model. Am J Physiol Renal Physiol 2009;297(6):F1668–77.
18. Celsi G, Kistner A, Aizman R, et al. Prenatal dexamethasone causes oligonephronia, sodium retention, and higher blood pressure in offspring. Pediatr Res 1998;44:317–22.
19. Ortiz LA, Quan A, Weinberg A, et al. Effect of prenatal dexamethasone on rat renal development. Kidney Int 2001;59(5):1663–9.
20. Ortiz LA, Quan A, Zarzar F, et al. Prenatal dexamethasone programs hypertension and renal injury in the rat. Hypertension 2003;41(2):328–34.
21. Wintour EM, Moritz KM, Johnson K, et al. Reduced nephron number in adult sheep, hypertensive as a result of prenatal glucocorticoid treatment. J Physiol 2003;549(Pt 3):929–35.
22. de Vries WB, van den Borne P, Goldschmeding R, et al. Neonatal dexamethasone treatment in the rat leads to kidney damage in adulthood. Pediatr Res 2010;67(1):72–6.
23. Aperia A, Broberger O, Elinder G, et al. Postnatal development of renal function in pre-term and full-term infants. Acta Paediatr Scand 1981;70(2):183–7.
24. Tsukahara H, Fujii Y, Tsuchida S, et al. Renal handling of albumin and beta-2-microglobulin in neonates. Nephron 1994;68(2):212–6.
25. Walker MW, Clark RH, Spitzer AR. Elevation in plasma creatinine and renal failure in premature neonates without major anomalies: terminology, occurrence and factors associated with increased risk. J Perinatol 2011;31(3):199–205.
26. Kent AL, Jyoti R, Robertson C, et al. Does extreme prematurity affect kidney volume at term corrected age? J Matern Fetal Neonatal Med 2009;22(5):435–8.
27. Schmidt IM, Chellakooty M, Boisen KA, et al. Impaired kidney growth in low-birth-weight children: distinct effects of maturity and weight for gestational age. Kidney Int 2005;68(2):731–40.
28. Drougia A, Giapros V, Hotoura E, et al. The effects of gestational age and growth restriction on compensatory kidney growth. Nephrol Dial Transplant 2009;24(1):142–8.
29. Huang HP, Tsai IJ, Lai YC, et al. Early postnatal renal growth in premature infants. Nephrology (Carlton) 2007;12(6):572–5.
30. Kandasamy Y, Smith R, Wright IM, et al. Extra-uterine renal growth in preterm infants: oligonephropathy and prematurity. Pediatr Nephrol 2013;28(9):1791–6.
31. Sutherland MR, Gubhaju L, Moore L, et al. Accelerated maturation and abnormal morphology in the preterm neonatal kidney. J Am Soc Nephrol 2011;22(7):1365–74.

32. Gubhaju L, Sutherland MR, Black MJ. Preterm birth and the kidney: implications for long-term renal health. Reprod Sci 2011;18(4):322–33.
33. Stelloh C, Allen KP, Mattson DL, et al. Prematurity in mice leads to reduction in nephron number, hypertension, and proteinuria. Transl Res 2012;159(2): 80–9.
34. Schreuder MF, Bueters RR, Huigen MC, et al. Effect of drugs on renal development. Clin J Am Soc Nephrol 2011;6(1):212–7.
35. Cullen L, Young R, Bertram J. Studies on the effects of gentamicin on rat metanephric development in vitro. Nephrology 2000;5:115–23.
36. Gilbert T, Gaonach S, Moreau E, et al. Defect of nephrogenesis induced by gentamicin in rat metanephric organ culture. Lab Invest 1994;70:656–66.
37. Gilbert T, Lelievre-Pegorier M, Merlet-Benichou C. Immediate and long-term renal effects of fetal exposure to gentamicin. Pediatr Nephrol 1990;4:445–50.
38. Gilbert T, Lelievre-Pegorier M, Malienou R, et al. Effects of prenatal and postnatal exposure to gentamicin on renal differentiation in the rat. Toxicology 1987;43(3):301–13.
39. Mallie JP, Gerard H, Gerard A. In-utero gentamicin-induced nephrotoxicity in rats. Pediatr Pharmacol (New York) 1986;5(4):229–39.
40. Nagai J, Takano M. Molecular aspects of renal handling of aminoglycosides and strategies for preventing the nephrotoxicity. Drug Metab Pharmacokinet 2004; 19(3):159–70.
41. Giapros V, Andronikou SK, Cholevas VI, et al. Renal function and effect of aminoglycoside therapy during the first ten days of life. Pediatr Nephrol 2003;18: 46–52.
42. Giapros VI, Andronikou SK, Cholevas VI, et al. Acute effects of gentamicin on glomerular and tubular functions in preterm neonates. Pediatr Nephrol 2006; 21(10):1389–92.
43. Langhendries JP, Battisti O, Bertrand J. Aminoglycoside nephrotoxicity and urinary excretion of N-acetyl-beta-D-glucosaminidase. Biol Neonate 1988;53: 253–9.
44. Nathanson S, Moreau E, Merlet-Benichou C, et al. In utero and in vitro exposure to beta-lactams impair kidney development in the rat. J Am Soc Nephrol 2000; 11(5):874–84.
45. Sáez F, Reverte V, Salazar F, et al. Hypertension and sex differences in the age-related renal changes when cyclooxygenase-2 activity is reduced during nephrogenesis. Hypertension 2009;53(2):331–7.
46. Kömhoff M, Wang JL, Cheng HF, et al. Cyclooxygenase-2-selective inhibitors impair glomerulogenesis and renal cortical development. Kidney Int 2000; 57(2):414–22.
47. Olliges A, Wimmer S, Nusing RM. Defects in mouse nephrogenesis induced by selective and non-selective cyclooxygenase-2 inhibitors. Br J Pharmacol 2011; 163(5):927–36.
48. Norwood VF, Morham SG, Smithies O. Postnatal development and progression of renal dysplasia in cyclooxygenase-2 null mice. Kidney Int 2000;58(6): 2291–300.
49. Kaplan BS, Restaino I, Raval DS, et al. Renal failure in the neonate associated with in utero exposure to non-steroidal anti-inflammatory agents. Pediatr Nephrol 1994;8(6):700–4.
50. van der Heijden BJ, Carlus C, Narcy F, et al. Persistent anuria, neonatal death, and renal microcystic lesions after prenatal exposure to indomethacin. Am J Obstet Gynecol 1994;171(3):617–23.

51. Sener A, Smith FG. Glomerular and tubular responses to N(G)-nitro-L-arginine methyl ester are age dependent in conscious lambs. Am J Physiol Regul Integr Comp Physiol 2002;282(5):R1512–20.
52. Keller RL, Tacy TA, Fields S, et al. Combined treatment with a nonselective nitric oxide synthase inhibitor (l-NMMA) and indomethacin increases ductus constriction in extremely premature newborns. Pediatr Res 2005;58(6): 1216–21.
53. Kang NS, Yoo KH, Cheon H, et al. Indomethacin treatment decreases renal blood flow velocity in human neonates. Biol Neonate 1999;76(5):261–5.
54. Kent AL, Brown L, Broom M, et al. Increased urinary podocytes following indomethacin suggests drug-induced glomerular injury. Pediatr Nephrol 2012;27(7): 1111–7.
55. Kent AL, Maxwell LE, Koina ME, et al. Renal glomeruli and tubular injury following indomethacin, ibuprofen, and gentamicin exposure in a neonatal rat model. Pediatr Res 2007;62(3):307–12.
56. Sutherland MR, Yoder BA, McCurnin D, et al. Effects of ibuprofen treatment on the developing preterm baboon kidney. Am J Physiol Renal Physiol 2012; 302(10):F1286–92.
57. Kent AL, Douglas-Denton R, Shadbolt B, et al. Indomethacin, ibuprofen and gentamicin administered during late stages of glomerulogenesis do not reduce glomerular number at 14 days of age in the neonatal rat. Pediatr Nephrol 2009; 24(6):1143–9.
58. Hinchliffe SA, Lynch MR, Sargent PH, et al. The effect of intrauterine growth retardation on the development of renal nephrons. Br J Obstet Gynaecol 1992;99:296–301.
59. Mañalich R, Reyes L, Herrera M, et al. Relationship between weight at birth and the number and size of renal glomeruli in humans: a histomorphometric study. Kidney Int 2000;58:770–3.
60. Zimanyi MA, Denton KM, Forbes JM, et al. A developmental nephron deficit in rats is associated with increased susceptibility to a secondary renal injury due to advanced glycation end-products. Diabetologia 2006;49(4):801–10.
61. Makrakis J, Zimanyi MA, Black MJ. Retinoic acid enhances nephron endowment in rats exposed to maternal protein restriction. Pediatr Nephrol 2007;22(11): 1861–7.
62. Zohdi V, Moritz KM, Bubb KJ, et al. Nephrogenesis and the renal renin-angiotensin system in fetal sheep: effects of intrauterine growth restriction during late gestation. Am J Physiol Regul Integr Comp Physiol 2007;293(3): R1267–73.
63. Brenner BM, Chertow GM. Congenital oligonephropathy and the etiology of adult hypertension and progressive renal injury. Am J Kidney Dis 1994;23(2): 171–5.
64. Merlet-Bénichou C, Gilbert T, Muffat-Joly M, et al. Intrauterine growth retardation leads to a permanent nephron deficit in the rat. Pediatr Nephrol 1994;8(2): 175–80.
65. Bassan H, Trejo LL, Kariv N, et al. Experimental intrauterine growth retardation alters renal development. Pediatr Nephrol 2000;15:192–5.
66. White SL, Perkovic V, Cass A, et al. Is low birth weight an antecedent of CKD in later life? A systematic review of observational studies. Am J Kidney Dis 2009; 54(2):248–61.
67. Gubhaju L, Black MJ. The baboon as a good model for studies of human kidney development. Pediatr Res 2005;58(3):505–9.

68. Sutherland MR, Gubhaju L, Yoder BA, et al. The effects of postnatal retinoic acid administration on nephron endowment in the preterm baboon kidney. Pediatr Res 2009;65(4):397–402.
69. Diderholm B. Perintatal energy metabolism with reference to IUGR and SGA: studies in pregnant women and newborn infants. Indian J Med Res 2009;130: 612–7.
70. Behrman RE, Lees MH, Peterson EN, et al. Distribution of the circulation in the normal and asphyxiated fetal primate. Am J Obstet Gynecol 1970;108(6): 956–69.
71. Lang U, Baker RS, Braems G, et al. Uterine blood flow–a determinant of fetal growth. Eur J Obstet Gynecol Reprod Biol 2003;110(Suppl 1):S55–61.
72. Kiserud T. Physiology of the fetal circulation. Semin Fetal Neonatal Med 2005; 10(6):493–503.
73. Cox B, Kotlyar M, Evangelou AI, et al. Comparative systems biology of human and mouse as a tool to guide the modeling of human placental pathology. Mol Syst Biol 2009;5:279.
74. Woods L, Weeks D, Rasch R. Programming of adult blood pressure by maternal protein restriction: role of nephrogenesis. Kidney Int 2004;65(4):1339–48.
75. Keijzer-Veen MG, Schrevel M, Finken MJ, et al, Dutch POPS-19 Collaborative Study Group. Microalbuminuria and lower glomerular filtration rate at young adult age in subjects born very premature and after intrauterine growth retardation. J Am Soc Nephrol 2005;16(9):2762–8.
76. Bacchetta J, Harambat J, Dubourg L, et al. Both extrauterine and intrauterine growth restriction impair renal function in children born very preterm. Kidney Int 2009;76(4):445–52.
77. Poston L. Intergenerational transmission of insulin resistance and type 2 diabetes. Prog Biophys Mol Biol 2011;106(1):315–22.
78. Boney CM, Verma A, Tucker R, et al. Metabolic syndrome in childhood: association with birth weight, maternal obesity, and gestational diabetes mellitus. Pediatrics 2005;115(3):e290–6.
79. Kim SY, England JL, Sharma JA, et al. Gestational diabetes mellitus and risk of childhood overweight and obesity in offspring: a systematic review. Exp Diabetes Res 2011;2011:541308.
80. Aerts L, Holemans K, Van Assche FA. Maternal diabetes during pregnancy: consequences for the offspring. Diabetes Metab Rev 1990;6(3):147–67.
81. Van Assche FA, Holemans K, Aerts L. Long-term consequences for offspring of diabetes during pregnancy. Br Med Bull 2001;60:173–82.
82. Tran S, Chen YW, Chenier I, et al. Maternal diabetes modulates renal morphogenesis in offspring. J Am Soc Nephrol 2008;19(5):943–52.
83. Nelson RG, Morgenstern H, Bennet PH. Intrauterine diabetes exposure and the risk of renal disease in diabetic Pima Indians. Diabetes 1998;47:1489–93.
84. Davis EM, Peck JD, Thompson D, et al. Maternal diabetes and renal agenesis/dysgenesis. Birth Defects Res 2010;88:722–7.
85. Chen YW, Chenier I, Tran S, et al. Maternal diabetes programs hypertension and kidney injury in offspring. Pediatr Nephrol 2010;25(7):1319–29.
86. Villar J, Carroli G, Wojdyla D, et al, World Health Organization Antenatal Care Trial Research Group. Preeclampsia, gestational hypertension and intrauterine growth restriction, related or independent conditions? Am J Obstet Gynecol 2006;194(4):921–31.
87. Suppo de Souza Rugolo LM, Bentlin MR, Trindade CE. Preeclampsia: effect on the fetus and newborn. Neo Reviews 2011;12:e198–206.

88. Alexander BT. Placental insufficiency leads to development of hypertension in growth-restricted offspring. Hypertension 2003;41(3):457–62.
89. Tang JR, Karumanchi SA, Seedorf G, et al. Excess soluble vascular endothelial growth factor receptor-1 in amniotic fluid impairs lung growth in rats: linking preeclampsia with bronchopulmonary dysplasia. Am J Physiol Lung Cell Mol Physiol 2012;302(1):L36–46.
90. Vohr BR, Allan W, Katz KH, et al. Early predictors of hypertension in prematurely born adolescents. Acta Paediatr 2010;99(12):1812–8.
91. Ejerblad E, Fored CM, Lindblad P, et al. Obesity and risk for chronic renal failure. J Am Soc Nephrol 2006;17:1695–702.
92. Mallamaci F, Tripepi G. Obesity and CKD progression: hard facts on fat CKD patients. Nephrol Dial Transplant 2013;28(Suppl 4):iv105–8.
93. Nenov VD, Taal MW, Sakharova OV, et al. Multi-hit nature of chronic renal disease. Curr Opin Nephrol Hypertens 2000;9:85–97.
94. Abitbol CL, Chandar J, Rodriguez MM, et al. Obesity and preterm birth: additive risks in the progression of kidney disease in children. Pediatr Nephrol 2009; 24(7):1363–70.
95. Hoy WE, Bertram JF, Denton RD, et al. Nephron number, glomerular volume, renal disease and hypertension. Curr Opin Nephrol Hypertens 2008;17(3): 258–65.
96. Luyckx VA, Bertram JF, Brenner BM, et al. Effect of fetal and child health on kidney development and long-term risk of hypertension and kidney disease. Lancet 2013;382(9888):273–83.
97. Sutherland MR, Bertagnolli M, Lukaszewski MA, et al. Preterm birth and hypertension risk: the oxidative stress paradigm. Hypertension 2014;63(1):12–8.
98. Rakow A, Johansson S, Legnevall L, et al. Renal volume and function in school-age children born preterm or small for gestational age. Pediatr Nephrol 2008; 23(8):1309–15.
99. Zaffanello M, Brugnara M, Bruno C, et al. Renal function and volume of infants born with a very low birth-weight: a preliminary cross-sectional study. Acta Paediatr 2010;99(8):1192–8.
100. Kwinta P, Klimek M, Drozdz D, et al. Assessment of long-term renal complications in extremely low birth weight children. Pediatr Nephrol 2011;26(7): 1095–103.
101. Keijzer-Veen MG, Devos AS, Meradji M, et al. Reduced renal length and volume 20 years after very preterm birth. Pediatr Nephrol 2010;25:499–507.
102. Rodríguez-Soriano J, Aguirre M, Oliveros R, et al. Long-term renal follow-up of extremely low birth weight infants. Pediatr Nephrol 2005;20:579–84.
103. Iacobelli S, Loprieno S, Bonsante F, et al. Renal function in early childhood in very low birthweight infants. Am J Perinatol 2007;24(10):587–92.
104. Keijzer-Veen MG, Kleinveld HA, Lequin MH, et al. Renal function and size at young adult age after intrauterine growth restriction and very premature birth. Am J Kidney Dis 2007;50(4):542–51.
105. Vanpée M, Blennow M, Linné T, et al. Renal function in very low birth weight infants: normal maturity reached during early childhood. J Pediatr 1992; 121(5 Pt 1):784–8.
106. Kistner A, Celsi G, Vanpee M, et al. Increased blood pressure but normal renal function in adult women born preterm. Pediatr Nephrol 2000;15(3–4):215–20.
107. Barker DJP, Osmond C. Infant mortality, childhood, nutrition, and ischaemic heart disease in England and Wales. Lancet 1986;1(8489):1077–81.

REVIEW 2:

PRETERM BIRTH AND/OR FACTORS THAT LEAD TO PRETERM DELIVERY: EFFECTS ON THE NEONATAL KIDNEY

CHAPTER ONE (REVIEW 2) DECLARATION

Declaration by candidate
[It is to be noted that I have used my married surname (Ryan) in this publication]

Chapter 1- Review 2 was published in the Journal of Neonatal Biology in 2015. Reprinted in this thesis is a copy of the final published manuscript. Ryan and Black et al. (2015). "Preterm Birth and/or Factors that Lead to Preterm Delivery: Effects on the Neonatal Kidney". J Neonatal Biol **4** (1).

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

Nature of contribution	Extent of contribution (%)
Wrote the manuscript	90 %

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

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
M. Jane Black	Assisted with editing the manuscript	N

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

Candidate's Signature	[redacted]	Date 21.9.16
Main Supervisor's Signature		Date 21.9.16

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Review Open Access

Preterm Birth and/or Factors that Lead to Preterm Delivery: Effects on the Neonatal Kidney

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Abstract

Preterm birth (defined as birth prior to 37 completed weeks of gestation), occurs in approximately 10% of all births and is one of the leading causes of neonatal morbidity and mortality worldwide. Preterm infants are born at a time when kidney development is still ongoing, and consequently can lead to renal impairment (in both the short-term and long-term), as well as severe glomerular abnormalities in some preterm infants. Since the glomerular abnormalities are not present in all preterm kidneys, this suggests that it is not preterm birth per se that leads to the glomerular abnormalities but may relate to factors associated with the etiology of the premature delivery, or factors in neonatal care. In this review, we provide an overview of what is currently known of how prenatal and postnatal factors can potentially impact on the immature kidneys of infants born preterm.

Keywords: Preterm birth; Intrauterine growth restriction; Kidney; Nephrogenesis; Neonatal care

Introduction

Preterm birth occurs in approximately 10% of all births and is one of the leading causes of neonatal morbidity and mortality worldwide. Preterm infants are born at a time when their organ systems are immature and hence, being born early can lead to adverse effects on organ structure and function both in the short-term and in the long-term. Preterm birth can lead to renal impairment in the neonatal period and can lead to glomerular abnormalities in some preterm infants. Since the glomerular abnormalities are not present in all preterm kidneys, this suggests that it is not preterm birth per se that leads to the glomerular abnormalities but may relate to factors associated with the etiology of the premature delivery or factors in neonatal care. Indeed, the etiology of preterm birth is multifactorial and the neonatal care of preterm infants is different for all individuals, depending on their postnatal sequelae. In this review, we provide an overview of what is currently known of how prenatal and postnatal factors can potentially impact on the immature kidneys of infants born preterm.

Preterm Birth

Preterm birth occurs in approximately 10% of all births and is one of the leading causes of neonatal morbidity and mortality worldwide [1]. Preterm birth is defined as birth prior to 37 completed weeks of gestation, with birth between 38-42 weeks of gestation considered as full term [2]. Preterm birth can be further sub-classified into moderately preterm, very preterm and extremely preterm. Moderately preterm infants are classified as those born between 32 to 36 weeks of gestation, very preterm births are those born between 28 and 31 weeks gestation, extremely preterm births are those born before28 weeks gestation [3]. Babies born prior to 23 weeks usually do not survive. The majority (60-70%) of preterm newborns are born between 34 and 36 weeks of gestation. The incidence of preterm infants born at 32-33 weeks gestation is ~20% and ~15% are born at 28-31 weeks, preterm birth prior to 28 weeks is the least common [3].

The global number of preterm deliveries each year has been slowly increasing and at the present time it is around 10% of births worldwide [4]. In the USA the incidence of preterm birth is 12.3% [5], in Europe it is 5-7% [4], and in Australia it is 8.2% [6]. However, within these populations some ethnic groups have a higher incidence of preterm birth. For example in African Americans the incidence of preterm birth is high at 17.5% [7] and in Indigenous Australians 13.3% of all births are preterm [6]. Of concern, the prevalence of preterm birth in developing countries is very high; for example, up to 17.5% of the reported birth sin South Africa are preterm and this is likely to be even higher as many births are not recorded [4].

Survival following preterm birth (especially in those born very, extremely preterm) has improved dramatically since the first introduction of neonatal intensive care units (in the 1960s). With subsequent refinements in prenatal and neonatal care, newborns born as early as 25 weeks gestation now have a 80% chance of survival [8,9]. In particular, the use of antenatal/neonatal corticosteroids (which accelerate lung maturation in the newborn) and surfactant therapy (which reduces alveolar surface tension in the presence of respiratory distress syndrome) have facilitated the recent improvement in survival [10].

The cause of premature delivery is multifactorial and differs with each pregnancy. It can occur spontaneously or be the result of emergency induced delivery. The most common identified causes of spontaneous preterm delivery are onset of premature labour (45%), and premature pre-labour rupture of the membranes (25%) [3]. The main identified cause of emergency induced delivery is maternal and fetal infection (35%) [3]. To date, the etiological mechanisms leading to spontaneous preterm labour and premature pre-labour rupture of the membranes are not well defined. There are a number of risk factors associated with increased risk of preterm delivery [3]. Pregnancy complications that often lead to emergency induced

preterm delivery include: Chorioamnionitis, placental insufficiency/abruption, pre-eclampsia, oligohydramnios (abnormal amniotic fluid levels) and intrauterine growth restriction (IUGR); IUGR is often a co-morbidity of these other pregnancy complications.

Although there has been a marked improvement in the survival of preterm infants over recent decades, preterm birth still remains the leading cause of infant mortality and morbidity. Perinatal mortality is currently around 6 to 8.5 times higher in preterm infants than in term infants [11]. Preterm infants are vulnerable to many postnatal complications due to the increased functional demands in the extra-uterine environment, at a time when the immature organs are ill-equipped for the functional transition to life ex-utero.

The increasing awareness of the potential adverse effects of being born early to immature organ systems has led to many studies over recent years looking at the consequences of preterm birth on fetal organ development, such as in the lungs [12], brain [13,14], gastrointestinal tract [15], and the kidney [16-19]. The effects of preterm birth in the neonatal kidney form the focus of this review.

Preterm Birth and its Effects on Renal Function and Nephrogenesis

Renal function

In the case of preterm infants, they are delivered at a time when nephrogenesis is often ongoing. In preterm neonates glomerular filtration rate (GFR) is very low at birth, and does not rise as rapidly as full term infants during the neonatal period [20,21]. As expected, glomerular filtration rate has shown to increase more rapidly after 34 weeks gestation [22,23] which coincides with the timing of the completion of nephrogenesis. Numerous studies have shown that preterm birth can lead to a high incidence of renal dysfunction in the neonate and under severe circumstances this can lead to renal failure [24,25]. The incidence of renal impairment in preterm infants is difficult to clearly define given that the kidneys are very immature at the time of birth. Hence, renal function is quite different in the preterm infant when compared to the term infant and many of these differences are due to immaturity rather than an underlying impairment. Certainly, both glomerular and tubular function are influenced by gestational age at birth and hence, it is difficult to establish whether the differences in renal function in preterm infants compared to term infants are solely due to underdevelopment of the nephrons or the result of injury in an immature kidney. During the first week after birth, glomerular filtration rate (GFR) is significantly lower in preterm infants compared to term infants [26-28] and it is positively correlated with gestational age at birth and postnatal age [29-31]. Likewise, creatinine clearance, one of the most commonly used markers of renal function, is positively correlated with both gestational age and postnatal age [20,21,29-39]. In addition, preterm neonates excrete high amounts of sodium in the early neonatal period compared to term neonates, with the fractional excretion of sodium inversely correlated with gestational age and postnatal age [29,39-43].

The presence of high levels of protein in the urine is indicative of pathological proteinuria (urine total protein ≥ 500 mg/l) and can be glomerular and/or tubular in origin. Specifically, the presence of proteins with a high molecular weight (albumin) in the urine, is indicative of a disruption in the integrity of the glomerular filtration barrier [44]. Alternatively, high levels of low molecular weight proteins (such as β2-microglobulin) are indicative of reduced reuptake by the proximal tubule cells [45,46]. The occurrence of proteinuria in neonates is strongly linked to gestational age at birth with studies in preterm infants reporting significantly greater albumin and β2-microglobulin concentrations over the first month of life in infants born <32 weeks gestation, compared to neonates born >32 weeks gestation [39,47]. To date, it remains unclear whether the observed proteinuria in preterm infants is a result of their renal immaturity or due to postnatal renal injury.

Acute kidney injury (previously defined as acute renal failure) is reported to occur in 8% to 24% of preterm infants admitted to neonatal intensive care units [24,48]. The mortality amongst these infants that are born <32 weeks gestation has been reported to be as high as 30-60% [49]. Acute kidney injury is defined as a sustained extreme decline in creatinine clearance; the initial clinical symptoms are a marked increase in serum creatinine and/or a sustained very low urine output [50,51]. The major risk factors for acute kidney injury are very low gestational age and low birth weight [52]. Other factors that have been linked to acute kidney injury are: hypotention, hypoxia, sepsis, maternal and neonatal drug administration (NSAIDs, indomethacin, antibiotics and vasopressor), a low apgar score, intraventricular haemorrhage (grade III and IV), necrotising enterocolitis, patent ductus arteriosus, respiratory distress syndrome, clinical interventions (intubation at birth),catheterization, phototherapy, and mechanical ventilation [24,52,53]. Of concern, mortality rates were reported to be significantly higher in neonates with renal dysfunction/renal failure [52].

In addition, to the short-term effects in the kidney, preterm birth is reported to influence long-term renal function [54-56]. For example, Rodriguez et al. [57], found GFR to be significantly lower in children ranging in age between 6.1 and 12.4 years who were born preterm, with evidence of renal injury (defects in tubular transport of phosphate) [57]. Iacobelli et al. [56], found that microalbuminaria was present in 8.3% of children examined that were born premature, ranging from 6-8 years of age. Similar findings were reported in a study of young adults; a lower GFR, higher serum creatinine and microalbuminaria was reported at 19 years of age in subjects born at <32 weeks gestation (and also small for gestational age) [54]. Furthermore, there is strong epidemiological evidence to link premature birth with the development of hypertension [58-63] and increased cardiovascular risk during adulthood [64,65]. This 'risk' may be further exacerbated in the presence of impaired renal function, possibly leading to hypertension, and the possible development of cardiovascular disease in later life [66].

Nephrogenesis

The nephrons are the functional units of the kidney and importantly, nephrogenesis (the formation of nephrons) is usually not completed until late in gestation (approximately 32 to 36 weeks gestation) [67]. Hence, the majority of preterm infants are born at a time when nephrogenesis is still ongoing. Over the past decade there have been a number of studies looking at the effect of preterm birth on nephrogenesis in the kidney. In the first of these studies, Rodriguez et al. [16] reported a reduced number of glomerular generations (thus implying reduced nephron endowment) in autopsied kidneys from babies that were born preterm compared to those born at term. However, in that study many of the preterm infants were also IUGR; hence, in that study interpretation of the findings is difficult, because it is well known that IUGR leads to reduced nephron endowment (see later section). Likewise, in another autopsy study the number of

glomerular generations was significantly reduced in preterm kidneys compared to terms [68]. Concomitant with these studies, we have also conducted a number of studies looking at the effect of premature birth in immature preterm kidneys, both in a non-human primate model of preterm birth and in autopsied kidneys of preterm infants. Our studies have convincingly shown that when nephrogenesis is ongoing in preterm infants at the time of birth, that nephrogenesis continues after birth; new nephrons are formed in the extra-uterine environment [17,19]. In our baboon studies, where the timing of nephrogenesis is similar to the human, we have shown that the kidneys are significantly larger in the preterm neonates, with a concomitant decrease in glomerular density, but nephron number was in the normal range (thus implying changes in tubular growth) [17]. Of concern, however, there was a high proportion of abnormal glomeruli (up to 18% in some kidneys) in the outer renal cortex in some of the preterm neonates. The abnormal glomeruli exhibited a shrunken immature glomerulus and an enlarged cystic Bowman's space. Similarly, in studies conducted in autopsied kidneys from infants born preterm [19], there was also an increase in kidney weight relative to body weight (probably due to the increased postnatal functional demands) and importantly new glomerular generations were formed after birth. However, there was a reduced nephrogenic zone width and a reduction in the proportion of glomeruli in the most immature stages (vesicle, comma-shaped, S-shaped and capillary loop stages), when compared to gestational age-matched controls, suggesting that cessation of nephrogenesis may be accelerated, and nephrogenic potential adversely impacted upon. To date, there have been no studies that have looked at exactly when nephrogenesis ceases in the preterm infant relative to gestational age-matched infants.

Alarmingly, as seen in the preterm baboon kidneys, there was a high proportion of abnormal glomeruli (with shrunken glomerular tufts and an enlarged cystic Bowman's space) in the outer renal cortex (up to 13% of glomeruli) in some of the preterm human kidneys [19]. A representative example of an abnormal glomerulus in the preterm human kidney is shown in Figure 1. Given the severity of these glomerular abnormalities it is unlikely that these glomeruli will ever be functional. Hence, our findings suggest that in these preterm infants the endowment of functional nephrons is adversely impacted upon by preterm birth, thereby affecting renal function both in the early postnatal period and later in life. To date, the causes of the glomerular abnormalities in the preterm kidneys are unknown. Given that not all preterm kidneys exhibit abnormal glomeruli, it is likely that it may be factors in the intrauterine environment (that lead to preterm delivery) that have adversely impacted upon the developing glomeruli or alternatively, it may be factors in the extra-uterine environment (haemodynamic and in the postnatal care) that have led to these glomerular abnormalities (Figure 2). In addition, there may be intrauterine factors and/or extra-uterine factors that adversely impact on nephrogenesis without inflicting glomerular pathologies. In the next sections, factors in the intrauterine environment (linked to the induction of preterm birth) and in the extra-uterine environment that could potentially adversely impact on the developing kidney are discussed.

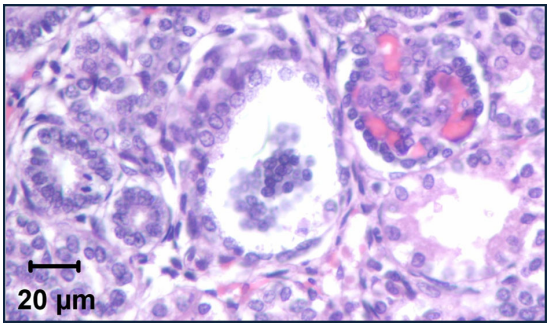

Figure 1: Representative photomicrograph of a preterm human kidney, exhibiting an abnormal glomerulus with an enlarged Bowman's space and shrunken immature glomerular cells. These abnormal glomeruli were only found in the outer renal cortex of the preterm human kidney, suggesting that they were formed in the extra-uterine environment. Scale bar 20 µm, stained with Haematoxylin and Eosin.

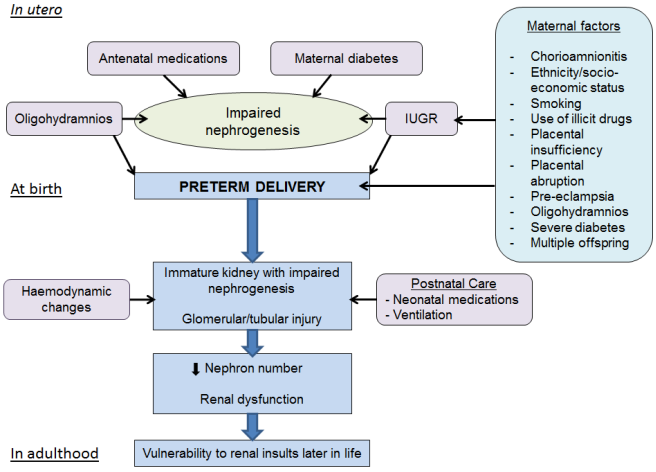

Figure 2: Flow diagram showing the factors associated with the etiology of premature delivery and factors in neonatal care that can potentially adversely impact upon the developing kidney. This in turn, can lead to impaired nephrogenesis and/or glomerular/tubular injury in the preterm neonate, and subsequent reduction in the number of functional nephrons at the beginning of life, leading to long-term vulnerability to renal disease.

Factors that can Potentially Impact on the Development of the Immature Kidney

Intrauterine factors

It is now well recognised that the in utero environment can directly influence organ structure and development. Hence, it is likely that the factors that lead to the induction of preterm delivery (spontaneous or assisted) can potentially impact on nephrogenesis and/or render the

kidneys vulnerable to premature delivery and subsequent pathology. In the next sections we describe some of the common factors/ conditions associated with preterm birth and how these factors can adversely impact on the development of the fetal kidney.

Intrauterine infection and inflammation (chorioamnionitis)

Intrauterine infection (in particular, chorioamnionitis) is widely acknowledged as a major contributor to premature delivery [13,69], especially in births prior to 32 weeks gestation [69,70]. A recent study by Ogge et al. [71], found that chronic chorioamnionitis was involved in 34% of the premature deliveries relating to preterm labour with intact membranes and 39% of preterm labour with membrane rupture. Chorioamnionitis is defined as inflammation of the chorion and amnion, caused by a bacterial infection which typically ascends from the vagina [3]. Importantly, chorioamnionitis can lead to fetal inflammatory response syndrome (FIRS) [72], and this has been shown to adversely influence neonatal organ development. The effect of exposure to inflammation in utero on the fetal kidney has recently been examined in fetal sheep [73,74]. In the study by Galinsky et al. [73] , there was a 20% reduction in nephron number, without any effect on body weight, when chorioamnionitis was induced in late gestation, using an acute intra-amniotic bolus dose of lipopolysaccharide (LPS, which initiates an inflammatory response similar to that observed with chorioamnionitis). Interestingly, however, when fetal lambs were exposed to a lower dose of LPS over a chronic period, during the period in gestation when nephrogenesis is rapidly ongoing, there were no observable detrimental effects on nephrogenesis [74]. Hence, it appears that with chronic low dose exposure that the kidney may be able to adapt, to prevent adverse effects on nephron formation. The contrasting findings from these two studiesdemonstrate that the timing, duration and extent of infection/ inflammation are important factors when assessing the impact of chorioamnionitis on the developing kidney.

Maternal diabetes

Exposure to intrauterine maternal diabetes can significantly influence fetal growth throughout gestation and lead to an early onset to preterm birth; this is of concern given the recent rise in Type 1 and type 2 and/or gestational diabetes [75,76]. A common consequence of intrauterine exposure to maternal diabetes is macrosomia, in particular asymmetric macrosomia [77]. Macrosomia oftenleads to exaggerated fetal growth, whereby the baby is born with a birth weight that is high for gestational age [78]. This increase in body weight is a result of excessive amounts of glucose and other nutrients crossing the placenta leading to an increase in fetal body growth. In contrast, when maternal diabetes (both Type 1 and Type 2) is severe, this can lead to IUGR in the infant [76,79]; the impacts of IUGR on the kidney are described later. With the increased prevalence of maternal diabetes there have been a number of recent studies looking at the effects on the fetal kidney. In a study conducted in preterm and term babies born to Pima Indian mother, exposure to maternal diabetes (Type 2 diabetes) during pregnancy led to a higher excretion of albumin (3.8 times higher) when compared to infants of pre-diabetic and non-diabetic mothers; thus indicative of renal injury in offspring exposed to diabetes in utero [80].

Animal studies, have reported an increased incidence of renal malformations in offspring born to diabetic mothers (Type 1 diabetes) [81,82]. In particular it has been shown that exposure to maternal diabetes can adversely impact nephrogenesis, with the offspring of diabetic mothers reported to have significantly smaller kidney and glomerular size, accompanied with a 40% reduction in nephron endowment [82]. The offspring of the diabetic mothers were significantly smaller in body weight, but there was no difference in kidney weight adjusted for body weight, compared to offspring of non-diabetic mothers [82]. Of concern, exposure to diabetes in utero led to greater glomerular and tubular apoptosis, compared to offspring not exposed to diabetes with the level of hyperglycaemiaa strong determinant of the severity of the adverse effects observed in the kidneys [82].

It is important to note, that although many of the animal studies relate to induction of type 1 diabetes in the mothers, the findings in relation to fetal development are likely to be also relevant to maternal type 2 and gestational diabetes, where the developing infant in all cases of maternal diabetes is exposed to hyperglycemia.

Antenatal medications

In general, administration of medications during pregnancy is avoided wherever possible, due to potential adverse effects on the developing fetus. However, it is important to note that there are some medications which are specifically administered to women 'at risk' of delivering prematurely, and although these medications are considered safe, they have the potential to adversely impact on the developing fetal kidney. In this section, we describe what is currently known in relation to these routinely prescribed medications.

Glucocorticoids

When it is considered likely that a woman will deliver prematurely, she is routinely administered glucocorticoids, usually betamethasone or dexamethasone. These medications have been shown to accelerate the maturation of the fetal lungs and thus, enhance the survival of the infant at preterm delivery [83,84]. In addition to the effects in the newborn's lungs, the administration of glucocorticoids has also been observed to increase mean arterial blood pressure, renal blood flow and glomerular filtration rate [85-87] this in turn, has the potential to affect renal function.

The effect of glucocorticoids on the developing kidney has been studied in animal models including: the rat [88-90], sheep [91-93] and baboon [17,94]. The findings suggest that exposure to glucocorticoids can affect nephron endowment and renal maturation. In sheep studies, administration of glucocorticoids during pregnancy (over 26 -28 days gestation) has been shown to significantly reduce nephron endowment in the exposed offspring [95] and in the neonatal rat, a reduction in glomerular density was observed when dexamethasone was administered at a time of ongoing postnatal nephrogenesis [96]. In our laboratory, we have looked at the effects of administration of antenatal glucocorticoids in a preterm baboon model [17]. Encouragingly, administration of antenatal glucocorticoids did not appear to have any direct adverse effects on the developing kidney and nephron endowment was within the normal range [17]. However, there was a 9% increase in developed glomeruli in the renal cortex in the betamethasone-exposed neonates, and a reduction in the width of the nephrogenic zone when compared to age-matched gestational controls. This suggests that there is accelerated renal maturation in response to glucocorticoid exposure and this is in accordance with other studies that show accelerated organ maturation as a result of glucocorticoid exposure [94,97].

Antibiotics

Infants that deliver preterm are often pre-exposed to antibiotics in utero, with antibiotics often prescribed to pregnant women with chorioamnionitis. Importantly, in this regard, antibiotics such as the aminoglycosides can readily cross the placenta [98] and there have been a number of experimental studies linking antibiotics with impairment of nephrogenesis [99-102]. For instance, it has been shown that incubation of metanephroi in culture with gentamicin leads to decreased branching morphogenesis of the ureteric tree and thus reduced nephron formation [99]. In addition, administration of antibiotics to guinea pig and rat dams has been shown to lead to oligonephronia in the offspring [103].

Indomethacin

Another routinely administered medication to women 'at risk' of preterm birth is indomethacin. Indomethacin is a tocolytic drug, which functions to reduce prostaglandin synthesis; it is thereby highly effective at prolonging pregnancy [104]. Of concern, however, in rodent studies in utero exposure to indomethacin has been reported to reduce nephron endowment and reduce glomerular filtration [105,106].

Oligohydramnios

Oligohydramnios is characterised by reduced levels of amniotic fluid during pregnancy. It can manifest as a result of fetal renal injury, such as decreased renal blood flow and/or reduced renal perfusion, which ultimately leads to a reduction in the amount of fetal urine excretion and consequently, the amount of amniotic fluid [107]. Other renal causes that attribute to a reduction in amniotic fluid include congenital anomalies such as: renal agenesis, polycystic kidneys, multicystic dysplastic kidneys and uteral or urtheral obstruction rupture of membranes [108]. It is also suggested that oligohydramnios can also result from bacterial infection within the amniotic cavity (such as chorioamnionitis), causing redistribution of blood flow within the developing fetus. A reduction in amniotic fluid at birth is often indicative of renal insufficiency in the neonate [109]. In utero detection of oligohydramnios often leads to the assisted induction of preterm labour as oligohydramnios has been linked to a number of inauspicious pregnancy outcomes such as perinatal death, fetal distress labour, low birth weight and poor infant health at birth [110].

Intrauterine Growth Restriction (IUGR)

IUGR is defined as body growth below the 10th percentile for gestational age. IUGR is multifactorial in origin with maternal race, economic status, diet and lifestyle (which can be interlinked) and complications of pregnancy all associated with induction of IUGR (Figure 2). IUGR is often a co-morbidity of preterm birth and it is linked both to spontaneous and assisted premature deliveries. In many pregnancies, it is difficult to ascertain whether it is the underlying cause of the IUGR, or the poor in utero growth of the fetus that is the stimulus for spontaneous preterm delivery. Likewise, the developing kidney can be directly impacted upon by the factors leading to IUGR, or alternatively, it can be a direct corollary of the IUGR. Certainly, the general consensus of the findings from the literature would support the latter with IUGR (regardless of the underlying causes) linked to poor organ development in the fetus and concomitant impairment of kidney development [111,112]. In the next sections some of the common factors associated with IUGR are described, including their links with preterm delivery.

Maternal ethnicity/socio-economic status

Maternal race has been linked with premature delivery and IUGR [3,113,114]. For example in the USA, African and African American women have been shown to have a four times higher chance of delivering a premature newborn compared to other racial groups [3]. In addition, women from South Asia and the Indian subcontinent have very high rates of IUGR and low birth weight [3], whereas, women from East Asia and Hispanic regions have been shown to have lower rates of premature delivery. In Australia, Indigenous Australians have a much higher frequency of IUGR and preterm delivery (approximately twice that of non-indigenous Australians) [6,115]. It is important to note, that in many of these populations (where there is a high incidence of IUGR) there is also a low socioeconomic status. Hence, the underlying cause of the IUGR may be due to poor maternal nutrition, lifestyle insults and poor maternal health (all described below), rather than their ethnicity per se.

Maternal diet

Malnutrition is a common cause of IUGR in underdeveloped countries [116]. It can result by under nutrition (inadequate food intake) and/or restriction of specific key nutrients in the diet. For example, data from the Dutch famine during World War 2 found that children born to mothers that had limited food available (less than 1000 calories per day) over the majority of their pregnancy gave birth to babies that were small for gestational age [117]. In another large study conducted in 538 women who delivered term, it was shown that a reduced protein diet during pregnancy leads to low birth weight in the neonate [118]. Similarly, in rat studies, IUGR is consistently reported when rat dams are fed a low protein diet during pregnancy [119-122].

Maternal lifestyle

Maternal behaviours such as smoking, high alcohol consumption, and ingesting illicit drugs have all been recognised as contributors to the risk of IUGR and premature birth [123-127]. Cigarette smoking has been reported to increase the risk of premature rupture of the membranes, pregnancy bleeding and pre-term labour. In addition, maternal smoking has been identified as a major cause of IUGR in developed countries, contributing to as high as 40% of all cases of IUGR [116]. Smoking causes vascular changes in the mother that can lead to placental insufficiency and hypoxia in the fetus [128]. It has also been associated with the down-regulation of important miRNAs of the placenta, leading to newborns that are small for gestational age [128]. Furthermore, nicotine found in cigarettes has been shown to pass the placenta, thus exerting a direct negative effect on the growth of the fetus [128]. Importantly, Dejmek et al. [129] also showed that reduced birth weight in newborns of smoking mothers was dose-dependent (that is number of cigarettes smoked per day).

Consumption of alcohol and use of illicit drugs during pregnancy is also linked to increased risk of preterm birth. In a cohort of 3000 African American women, alcohol and cocaine use was found to be associated with extreme preterm birth [125]. Of particular concern, a study by O'Leary et al. [127], found that moderate ingestion of alcohol consumption (only during the first trimester of pregnancy) was associated with pre-term birth In Australia, the high rate of preterm

birth in the Indigenous community is thought to be attributed to high rates of tobacco, alcohol and drug use in pregnant women [130].

Placental insufficiency/abruption

The placenta is a vital organ that develops specifically during pregnancy to support the growth of the developing fetus. The role of the placenta is to supply the fetus with an adequate amount of nutrients and oxygen for normal fetal growth. In developed countries the most common cause of IUGR is placental insufficiency [131,132] and it is also strongly linked with preterm birth [133]. Placental insufficiency occurs when the placenta does not develop normally and thus it is unable to adequately support the developing baby. It is usually caused by reduced uterine artery blood flow (uteroplacental insufficiency) [131].

Placental abruption occurs in late gestation and is a serious condition where the placenta partially, or completely, separates from the lining of the uterus; the effects on the developing fetus depend on the severity [134]. The full separation of the placenta from the uterus lining can lead to in utero death and subsequent stillbirth, if the fetus is not delivered at the time of abruption. When there is partial placental separation the fetus is growth restricted and preterm birth will often ensue (spontaneous or assisted).

Pre-eclampsia

Pre-eclampsia is pregnancy-associated hypertension [135]; it is a multi-system disorder which affects approximately 8% of pregnancies [136]. It occurs when placentation is abnormal, which can cause the mother to experience intravascular coagulation, bleeding and organ failure (hepatic and renal) following poor perfusion. These complications subside with the delivery of the fetus. Severe pre-eclampsia can lead to maternal death and thus, it is a major cause of assisted preterm birth [137]. In addition, pre-eclampsia during pregnancy is a major risk factor for IUGR [138,139] as it usually results in placental insufficiency. Higher rates of pre-eclampsia are seen amongst women with pre-existing hypertension, diabetes mellitus or previous history of pre-eclampsia [140]. Of concern, there has been an increased incidence of pre-eclampsia in developing countries over recent years [141].

Multiple births

Pregnancies with multiple fetuses exhibit a higher risk of placental dysfunction and placental insufficiency. This is associated with the slowed growth rate of twins during late gestation when compared to the singleton growth rate [142]. The incidence of multiple births is increasing and this is largely attributed to the increase in availability of infertility treatment, such as ovulation induction [143,144]. Monochorionic twins (identical twins that share one placenta) have a much greater chance of being born IUGR than dichorionic twins (twins that do not share the same placenta) [145,146]. Discordant growth, results from unequal distribution of uteroplacental blood flow to the fetuses [147].

IUGR adversely Impacts on Nephron Endowment at Birth

It is now well established that IUGR, regardless of the etiological origins (many of these described above), can adversely impact on the number of nephrons formed within the developing kidney. Indeed there are many experimental studies that have shown that when IUGR is induced by maternal dietary manipulations, or by induction of placental insufficiency, that nephron number is reduced in the offspring [148-152]. In general, nephron endowment at birth is directly proportional to kidney size [17,153,154], so in the case of the IUGR infant the reduction in body size at birth is accompanied by a decrease in kidney size and in the number of nephrons. In support of this concept, in autopsied human kidneys there was a linear relationship between the number of glomeruli (and therefore nephrons) and birth weight in full term neonates[155]; neonates below the 10th percentile of birth weight had 30% fewer glomeruli than the neonates with birth weights above the 10th percentile[155].

However, it is important to note that the timing of the growth insult during gestation is important. If the growth restriction occurs late in gestation, when nephrogenesis is already complete, or close to completion, the number of nephrons formed within the kidney will not be affected by the IUGR, yet birth weight will be significantly reduced. For example, in a study performed in our laboratory [151], placental insufficiency was experimentally induced in fetal lambs late in gestation (from 120-140 days gestation; term is 147 days) at a time when nephrogenesis was nearing completion. This study revealed a significant decrease in body weight and kidney weight in response to IUGR compared to appropriately grown lambs. However, nephron endowment in the IUGR lambs was not different to the control lambs. In contrast, IUGR caused by twinning led to a significant reduction in nephron endowment [151].

Extra-uterine (Postnatal) factors

There are a number of factors in the postnatal environment (haemodynamic and factors associated with postnatal care), that can potentially adversely impact on the immature kidneys of the preterm infant. Some of the major ones are described below.

Change in haemodynamics

There is a major hemodynamic transition at the time of birth, when the circulatory dependence on the placenta is terminated and the in utero configuration of circulation is changed to the ex utero configuration [156]. In the immediate period following birth the kidneys need to rapidly adapt to the extra-uterine environment whereby they are now required to independently control fluid and electrolyte levels [20]. Following birth, there is also a significant increase in mean arterial pressure and cardiac output and a reduced renal vascular resistance facilitates an increase in renal blood flow [33]. Since resistance of the afferent and efferent arterioles is a determinant of glomerular capillary pressure the glomerular filtration rate also increases at birth and sodium reabsorption subsequently increases [157]. Hence, the immature kidneys of preterm infants are exposed to a marked increase in renal blood flow and blood pressure in the immediate neonatal period and this has the potential to lead to renal injury. To date, there is little information as to how changes in renal blood flow and pressure directly impact on nephrogenesis and on the recently formed immature nephrons in the preterm kidney. It is conceivable that increases in blood flow and blood pressure could lead to renal vascular injury and to the glomerular injury observed in preterm infants. In this regard, in future studies it will be important to look at the role of renal endothelial function in relation to prematurity. Certainly, endothelial dysfunction has been described in other organs following preterm birth and IUGR. Low birth weight and premature birth has been previously reported to cause endothelial dysfunction in the intestines, skin, retinal vessels and peripheral arteries [158]. Hence,

it is plausible to suggest that preterm birth and low birth weight could also affect developing arteries and capillaries in the immature kidney. It is imperative in future studies to address this.

Hyperoxia and ventilation

In utero, the fetus normally develops in a relatively hypoxic environment (5% oxygen) and this facilitates both vascular and tubular development in the kidney [159-161]. At birth, the neonate is exposed to an abrupt increase in oxygen from ~5% to 21% [161]. The blood oxygen saturation levels (SpO2) rise from 45-55% in the fetus [162] to 80-90% in the first five minutes after birth [163]. Hence, when a baby is born preterm, the immature kidney is no longer growing in an hypoxic environment and hence, it is likely that this will lead to deleterious effects on the growth of the renal vasculature and the tubules. This is an important area for future research and to our knowledge this has not been investigated.

In addition, in the preterm neonate the lungs are very immature at the time of birth; therefore, the neonate requires resuscitation and ongoing ventilation [164]. Exposure to supplemental oxygen therapies, such as ventilation, can lead to exposure to very high concentrations of O2 (up to 100%), in an attempt to normalise blood oxygen levels [165,166]. However, during this process the infant can experience high blood oxygen levels (often only transitory) until the blood oxygen levels become normalised. Of concern, hyperoxia can lead to oxidative stress of the neonate, which has been shown to subsequently cause cellular injury and cell death in response to accumulation of free radicals and thereby exhaustion of antioxidants[167,168]. Consequently, this can lead to a number of common morbidities of prematurity such as, retinopathy of prematurity, necrotizing enterocolitis and bronchopulmonary dysplasia [169]. In the kidney of the human neonate, oxidative stress has been reported to cause tubular injury [170] and it has been linked to impairment of nephrogenesis in animal studies [171]. In the rat model (where nephrogenesis is ongoing in the first two weeks after birth), a significant reduction of nephrons (25%) was reported in adulthood (25-35 weeks of age) [171] following exposure to 80% oxygen during the early postnatal period. In contrast, however, in a more recent study [172], exposure to 65% oxygen levels for seven days of postnatal life, did not appear to have any deleterious effects on nephrogenesis. However, in that study, the kidneys of the hyperoxia-exposed mice did exhibit glomerular hypertrophy in adulthood (postnatal day 56), suggestive of possible reduced renal functional capacity.

Neonatal medications

Preterm infants are administered many medications in the immediate period following birth; the treatment regime varies from infant to infant and is ultimately dependent on the clinical sequelae of each infant. Many of the medications administered to the infants are known to be toxic to the kidneys but their benefits to the infant outweigh the potential adverse effects on the kidneys. Some of the commonly administered drugs are: non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin and ibuprofen and aminoglycoside antibiotics (such as gentamicin). There are a number of experimental studies which demonstrate that treatment with these medications can have adverse effects on renal function in the postnatal period and lead to renal injury [173-176]

NSAIDS

In the preterm neonate, exposure to indomethacin after birth has been shown to lead to a significant increase in the concentration of podocytes in the urine, as well as increased urine albumin excretion [177], suggestive of renal injury. Treatment with NSAIDs is also linked to impaired renal function. A recent study of renal function in preterm babies indicated that NSAIDs administered to the preterm infant significantly reduced neonatal renal drug clearance, likely associated with a reduced GFR [178]. In the rodent model, postnatal administration of NSAIDs and/or gentamicin during the period of postnatal nephrogenesis is associated with a number of structural changes in immature rodent kidneys [102]. These changes include 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. In these studies early administration of indomethacin caused a significant reduction in nephron endowment at 14 days postnatal age in rats, however, these effects were not observed in the kidney when exposed to ibuprofen. In the mouse model, postnatal exposure to NSAIDs caused a significant reduction in glomerular density and glomerular and tubular volumes in the kidneys [179]. Importantly, in preterm baboons (born at a time, equivalent to ~27 week gestation in the human), administration of ibuprofen during the postnatal period caused a significant reduction in nephrogenic zone width [180]. This suggests that prostaglandin inhibition may result in the early cessation of nephrogenesis.

Antibiotics

It is known that antibiotics, such as the aminoglycosides, can be nephrotoxic in the newborn (with the preterm infant most vulnerable) [101] and they are also linked with impairment of nephrogenesis [103,173]. Administration of gentamicin in neonates has been shown to primarily result in renal tubular necrosis [100], which consequently leads to increased sodium excretion, proteinuria, and a significant reduction in GFR [101,181,182].

Preterm infants are often exposed to antibiotics in utero (see earlier section) and/or in the postnatal period when there is evidence of infection. In this regard, in a study of preterm human infants, using a multivariate logistic analysis, it was found that mothers of infants with acute renal failure received more drugs during pregnancy and delivery (mainly antibiotics and non-steroidal anti-inflammatory drugs) [183]. Moreover, in the first few days of life and before diagnosis of acute renal failure, the preterm infants that developed renal failure received more drugs (antibiotics, NSAIDs and diuretics) and for a longer period [183].

Postnatal Nutrition

Recent studies highlight the importance of postnatal nutrition on the growth and function of the kidney in IUGR and preterm infants. Certainly, when nephrogenesis is ongoing there are usually strong linear correlations between nephron number and kidney size [155]. Impaired growth after birth (extra-uterine growth restriction; EUGR) often occurs during the postnatal period in preterm infants [181]; hence, it is likely that impaired body growth in the immediate period after birth will adversely affect kidney growth and nephron endowment in the preterm infant. Therefore, there is the potential for improved postnatal nutrition to positively impact on the number of nephrons formed. In support of this idea, in a recent study of preterm

children (born <30 weeks gestation) [182] glomerular filtration rate was significantly decreased (suggestive of reduced nephron endowment) at 7 years of age, in those that were either intra or extra-uterine growth restricted. Importantly, the extra-uterine growth restricted children were found to have significantly lower protein-energy intake during their first week of life when compared to IUGR or appropriately grown children. In addition, Schmidt et al. [183] observed that consuming protein-rich formula, compared to just breast milk, during the early postnatal period caused a significant increase in kidney size.

Conclusion

This review highlights the many factors associated with the etiology of preterm birth and in the postnatal environment that can potentially impact on the immature kidney of the preterm infant. In order to improve long-term renal health in subjects born preterm, it is now important in future studies, to develop interventional strategies that mitigate the adverse impact of the intrauterine and extra-uterine environment on the immature kidney. At this stage, there is no clear indicator of the causes of the glomerular abnormalities associated with preterm birth. Carefully controlled animal studies can help to elucidate the causes of the glomerular abnormalities and this is an important area of future research. In regards to renal injury, this review highlights a number of medications, commonly used in the neonatal intensive care unit that can lead to renal impairment. Hence, it is the challenge for the neonatologist, when deciding to use these medications, to ascertain whether the benefits outweigh the risks.

References

1. Blencowe H, Cousens S, Oestergaard MZ, Chou D, Moller AB, et al. (2012) National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications. Lancet 379: 2162-2172.
2. Tucker J, Mc Guire W (2004) Epidemiology of preterm birth. BMJ 329: 675-678.
3. Goldenberg RL, Culhane JF, Iams JD, Romero R (2008) Epidemiology and causes of preterm birth. Lancet 371: 75-84.
4. Beck S, Wojdyla D, Say L, Betran AP, Merialdi M, et al. (2010) The worldwide incidence of preterm birth: a systematic review of maternal mortality and morbidity. Bull World Health Organ 88: 31-38.
5. Mathews TJ, Miniño AM, Osterman MJ, Strobino DM, Guyer B (2011) Annual summary of vital statistics: 2008. Pediatrics 127: 146-157.
6. Laws Pj, Li Z, Sullivan EA (2010) Australia's mothers and babies 2008. Perinatal statistics, Australian Institute of Health and Welfare, Australia.
7. Martin JA, Hamilton BE, Ventura SJ, Osterman MJ, Kirmeyer S, et al. (2011) Births: final data for 2009. Natl Vital Stat Rep 60: 1-70.
8. Allen MC, Donohue PK, Dusman AE (1993) The limit of viability--neonatal outcome of infants born at 22 to 25 weeks' gestation. N Engl J Med 329: 1597-1601.
9. Kutz P, Horsch S, Kühn L, Roll C (2009) Single-centre vs. population-based outcome data of extremely preterm infants at the limits of viability. Acta Paediatr 98: 1451-1455.
10. Roberts D, Dalziel S (2006) Antenatal corticosteroids for accelerating fetal lung maturation for women at risk of preterm birth. Cochrane Database Syst Rev : CD004454.
11. Pulver LS, Guest-Warnick G, Stoddard GJ, Byington CL, Young PC (2009) Weight for gestational age affects the mortality of late preterm infants. Pediatrics 123: e1072-1077.
12. Kallapur SG, Ikegami M (2006) Physiological consequences of intrauterine insults. Paediatr Respir Rev 7: 110-116.
13. Goldenberg RL, Hauth JC, Andrews WW (2000) Intrauterine infection and preterm delivery. N Engl J Med 342: 1500-1507.
14. De Felice C, Toti P, Laurini RN, Stumpo M, Picciolini E, et al. (2001) Early neonatal brain injury in histologic chorioamnionitis. J Pediatr 138: 101-104.
15. Kafetzis DA, Skevaki C, Costalos C (2003) Neonatal necrotizing enterocolitis: an overview. Curr Opin Infect Dis 16: 349-355.
16. Rodríguez MM, Gómez AH, Abitbol CL, Chandar JJ, Duara S, et al. (2004) Histomorphometric analysis of postnatal glomerulogenesis in extremely preterm infants. Pediatr Dev Pathol 7: 17-25.
17. Gubhaju L, Sutherland MR, Yoder BA, Zulli A, Bertram JF, et al. (2009) Is nephrogenesis affected by preterm birth? Studies in a non-human primate model. Am J Physiol Renal Physiol 297: 1668-1677.
18. Gubhaju L, Sutherland MR, Black MJ (2011) Preterm birth and the kidney: implications for long-term renal health. Reprod Sci 18: 322-333.
19. Sutherland MR, Gubhaju L, Moore L, Kent AL, Dahlstrom JE, et al. (2011) Accelerated maturation and abnormal morphology in the preterm neonatal kidney. J Am Soc Nephrol 22: 1365-1374.
20. Aperia A, Broberger O, Elinder G, Herin P, Zetterström R (1981) Postnatal development of renal function in pre-term and full-term infants. Acta Paediatr Scand 70: 183-187.
21. Bueva A, Guignard JP (1994) Renal function in preterm neonates. Pediatr Res 36: 572-577.
22. Arant BS Jr (1978) Developmental patterns of renal functional maturation compared in the human neonate. J Pediatr 92: 705-712.
23. Satlin LM, Woda CB, Schwartz GJ: Development of function in the metanephric kidney; in Vize PD, Woolf A, Bard JBL (eds): The kidney: From normal development to congenital disease. Sydney, Academic Press, 2003
24. Stapleton FB, Jones DP, Green RS (1987) Acute renal failure in neonates: incidence, etiology and outcome. Pediatr Nephrol 1: 314-320.
25. Choker G, Gouyon JB (2004) Diagnosis of acute renal failure in very preterm infants. Biol Neonate 86: 212-216.
26. Siegel SR, Oh W (1976) Renal function as a marker of human fetal maturation. Acta Paediatr Scand 65: 481-485.
27. Finney H, Newman DJ, Thakkar H, Fell JM, Price CP (2000) Reference ranges for plasma cystatin C and creatinine measurements in premature infants, neonates, and older children. Arch Dis Child 82: 71-75.
28. Schreuder MF, Wilhelm AJ, Bökenkamp A, Timmermans SM, Delemarre-van de Waal HA, et al. (2009) Impact of gestational age and birth weight on amikacin clearance on day 1 of life. Clin J Am Soc Nephrol 4: 1774-1778.
29. Clark PM, Bryant TN, Hall MA, Lowes JA, Rowe DJ (1989) Neonatal renal function assessment. Arch Dis Child 64: 1264-1269.
30. Gordjani N, Burghard R, Leititis JU, Brandis M (1988) Serum creatinine and creatinine clearance in healthy neonates and prematures during the first 10 days of life. Eur J Pediatr 148: 143-145.
31. Iacobelli S, Bonsante F, Ferdinus C, Labenne M, Gouyon JB (2009) Factors affecting postnatal changes in serum creatinine in preterm infants with gestational age <32 weeks. J Perinatol 29: 232-236.
32. Ross B, Cowett RM, Oh W (1977) Renal functions of low birth weight infants during the first two months of life. Pediatr Res 11: 1162-1164.
33. Fawer CL, Torrado A, Guignard JP (1979) Maturation of renal function in full-term and premature neonates. Helv Paediatr Acta 34: 11-21.
34. Sulyok E, Varga F, Györy E, Jobst K, Csaba IF (1979) Postnatal development of renal sodium handling in premature infants. J Pediatr 95: 787-792.
35. Wilkins BH (1992) Renal function in sick very low birthweight infants: 4. Glucose excretion. Arch Dis Child 67: 1162-1165.
36. Gallini F, Maggio L, Romagnoli C, Marrocco G, Tortorolo G (2000) Progression of renal function in preterm neonates with gestational age < or = 32 weeks. Pediatr Nephrol. 15:119-124.
37. Cuzzolin L, Fanos V, Pinna B, di Marzio M, Perin M, et al. (2006) Postnatal renal function in preterm newborns: a role of diseases, drugs and therapeutic interventions. Pediatr Nephrol 21: 931-938.

38. Thayyil S, Sheik S, Kempley ST, Sinha A (2008) A gestation- and postnatal age-based reference chart for assessing renal function in extremely premature infants. J Perinatol 28: 226-229.
39. Gubhaju L, Sutherland MR, Horne RS, Medhurst A, Kent AL, et al. (2014) Assessment of renal functional maturation and injury in preterm neonates during the first month of life. Am J Physiol Renal Physiol 307: 149-158.
40. Galaske RG (1986) Renal functional maturation: renal handling of proteins by mature and immature newborns. Eur J Pediatr 145: 368-371.
41. Tsukahara H, Yoshimoto M, Saito M, Sakaguchi T, Mitsuyoshi I, et al. (1990) Assessment of tubular function in neonates using urinary beta 2-microglobulin. Pediatr Nephrol 4: 512-514.
42. Fell JM, Thakkar H, Newman DJ, Price CP (1997) Measurement of albumin and low molecular weight proteins in the urine of newborn infants using a cotton wool ball collection method. Acta Paediatr 86: 518-522.
43. Awad H, el-Safty I, el-Barbary M, Imam S (2002) Evaluation of renal glomerular and tubular functional and structural integrity in neonates. Am J Med Sci 324: 261-266.
44. Mathieson PW (2004) The cellular basis of albuminuria. Clin Sci (Lond) 107: 533-538.
45. Tomlinson PA (1992) Low molecular weight proteins in children with renal disease. Pediatr Nephrol 6: 565-571.
46. Christensen EI, Birn H (2001) Megalin and cubilin: synergistic endocytic receptors in renal proximal tubule. Am J Physiol Renal Physiol 280: F562-573.
47. Tsukahara H, Fujii Y, Tsuchida S, Hiraoka M, Morikawa K, et al. (1994) Renal handling of albumin and beta-2-microglobulin in neonates. Nephron 68: 212-216.
48. Hentschel R, Lödige B, Bulla M (1996) Renal insufficiency in the neonatal period. Clin Nephrol 46: 54-58.
49. Andreoli SP (2004) Acute renal failure in the newborn. Semin Perinatol 28: 112-123.
50. Bellomo R, Ronco C, Kellum JA, Mehta RL, Palevsky P (2004) 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.
51. Mehta RL, Kellum JA, Shah SV, Molitoris BA, Ronco C, et al. (2007) Acute Kidney Injury Network: report of an initiative to improve outcomes in acute kidney injury. Crit Care 11: R31.
52. Walker MW, Clark RH, Spitzer AR (2011) Elevation in plasma creatinine and renal failure in premature neonates without major anomalies: Terminology, occurrence and factors associated with increased risk. J Perinatol. 31:199-205.
53. Cataldi L, Leone R, Moretti U, De Mitri B, Fanos V, et al. (2005) Potential risk factors for the development of acute renal failure in preterm newborn infants: a case-control study. Arch Dis Child Fetal Neonatal Ed 90: F514-519.
54. Keijzer-Veen M, Schrevel M, Finken M, Dekker F, Nauta J, et al. (2005) Microalbuminuria and lower glomerular filtration rate at young adult age in subjects born very premature and after intrauterine growth retardation. Journal of the American Society of Nephrology 16: 2762-2768.
55. Rodríguez-Soriano J, Aguirre M, Oliveros R, Vallo A (2005) Long-term renal follow-up of extremely low birth weight infants. Pediatr Nephrol 20: 579-584.
56. Iacobelli S, Loprieno S, Bonsante F, Latorre G, Esposito L, et al. (2007) Renal function in early childhood in very low birthweight infants. Am J Perinatol 24: 587-592.
57. Rodriguez MM, Gomez A, Abitbol C, Chandar J, Montané B, et al. (2005) Comparative renal histomorphometry: a case study of oligonephropathy of prematurity. Pediatr Nephrol 20: 945-949.
58. Siewert-Delle A, Ljungman S (1998) The impact of birth weight and gestational age on blood pressure in adult life: a population-based study of 49-year-old men. Am J Hypertens 11: 946-953.
59. Irving RJ, Belton NR, Elton RA, Walker BR (2000) Adult cardiovascular risk factors in premature babies. Lancet 355: 2135-2136.
60. Kistner A, Celsi G, Vanpee M, Jacobson SH (2000) Increased blood pressure but normal renal function in adult women born preterm. Pediatr Nephrol 15: 215-220.
61. Bonamy AK, Bendito A, Martin H, Andolf E, Sedin G, et al. (2005) Preterm birth contributes to increased vascular resistance and higher blood pressure in adolescent girls. Pediatr Res 58: 845-849.
62. Bonamy AK, Martin H, Jörneskog G, Norman M (2007) Lower skin capillary density, normal endothelial function and higher blood pressure in children born preterm. J Intern Med 262: 635-642.
63. Cooper R, Atherton K, Power C (2009) Gestational age and risk factors for cardiovascular disease: evidence from the 1958 British birth cohort followed to mid-life. Int J Epidemiol 38: 235-244.
64. Ciccone MM, Scicchitano P, Zito A, Gesualdo M, Sassara M, et al. (2011) Different functional cardiac characteristics observed in term/preterm neonates by echocardiography and tissue doppler imaging. Early Hum Dev.87:555-558.
65. Mercuro G, Bassareo PP, Flore G, Fanos V, Dentamaro I, et al. (2013) Prematurity and low weight at birth as new conditions predisposing to an increased cardiovascular risk. Eur J Prev Cardiol 20: 357-367.
66. Brenner BM, Chertow GM (1994) Congenital oligonephropathy and the etiology of adult hypertension and progressive renal injury. Am J Kidney Dis 23: 171-175.
67. Hinchliffe SA, Sargent PH, Howard CV, Chan YF, Van Velzen D (1991) Human intrauterine renal growth expressed in absolute number of glomeruli assessed by the disector method and cavalieri principle. Lab Invest 64: 777-784.
68. Faa G, Gerosa C, Fanni D, Nemolato S, Locci A, et al. (2010) Marked interindividual variability in renal maturation of preterm infants: lessons from autopsy. J Matern Fetal Neonatal Med 23 Suppl 3: 129-133.
69. Mueller-Heubach E, Rubinstein DN, Schwarz SS (1990) Histologic chorioamnionitis and preterm delivery in different patient populations. Obstet Gynecol 75: 622-626.
70. Lahra MM, Jeffery HE (2004) A fetal response to chorioamnionitis is associated with early survival after preterm birth. Am J Obstet Gynecol 190: 147-151.
71. Oggé G, Romero R, Lee DC, Gotsch F, Than NG, et al. (2011) Chronic chorioamnionitis displays distinct alterations of the amniotic fluid proteome. J Pathol 223: 553-565.
72. Gantert M, Been JV, Gavilanes AW, Garnier Y, Zimmermann LJ, et al. (2010) Chorioamnionitis: a multiorgan disease of the fetus? J Perinatol 30 Suppl: S21-30.
73. Galinsky R, Moss TJ, Gubhaju L, Hooper SB, Black MJ, et al. (2011) Effect of intra-amniotic lipopolysaccharide on nephron number in preterm fetal sheep. Am J Physiol Renal Physiol 301: F280-285.
74. Ryan D, Atik A, De Matteo R, Harding R, Black MJ (2013) Chronic intrauterine exposure to endotoxin does not alter fetal nephron number or glomerular size. Clin Exp Pharmacol Physiol.
75. Hunt KJ, Schuller KL (2007) The increasing prevalence of diabetes in pregnancy. Obstet Gynecol Clin North Am 34: 173-199, vii.
76. Magon N, Chauhan M (2012) Pregnancy in Type 1 Diabetes Mellitus: How Special are Special Issues? N Am J Med Sci 4: 250-256.
77. Aerts L, Holemans K, Van Assche FA (1990) Maternal diabetes during pregnancy: consequences for the offspring. Diabetes Metab Rev 6: 147-167.
78. Jovanovic L (2001) What is so bad about a big baby? Diabetes Care 24: 1317-1318.
79. Rowan JA, Luen S, Hughes RC, Sadler LC, McCowan LM (2009) Customised birthweight centiles are useful for identifying small-for-gestational-age babies in women with type 2 diabetes. Aust N Z J Obstet Gynaecol 49: 180-184.

80. Nelson RG, Morgenstern H, Bennett PH (1998) Intrauterine diabetes exposure and the risk of renal disease in diabetic Pima Indians. Diabetes 47: 1489-1493.
81. Zhang SL, Chen YW, Tran S, Chenier I, Hébert MJ, et al. (2007) Reactive oxygen species in the presence of high glucose alter ureteric bud morphogenesis. J Am Soc Nephrol 18: 2105-2115.
82. Tran S, Chen YW, Chenier I, Chan JS, Quaggin S, et al. (2008) Maternal diabetes modulates renal morphogenesis in offspring. J Am Soc Nephrol 19: 943-952.
83. Shanks A, Gross G, Shim T, Allsworth J, Sadovsky Y, et al. (2010) Administration of steroids after 34 weeks of gestation enhances fetal lung maturity profiles. Am J Obstet Gynecol 203: 47.
84. Crowther CA, McKinlay CJ, Middleton P, Harding JE (2011) Repeat doses of prenatal corticosteroids for women at risk of preterm birth for improving neonatal health outcomes. Cochrane Database Syst Rev: CD003935.
85. al-Dahan J, Stimmler L, Chantler C, Haycock GB (1987) The effect of antenatal dexamethasone administration on glomerular filtration rate and renal sodium excretion in premature infants. Pediatr Nephrol. 1:131-135.
86. Kari M, Hallma M, Eronen M, Teramo K, Virtanen M, et al. (1994) Prenatal dexamethasone treatment in conjunction with rescue therapy of human surfactant: A randomized placebo-controlled multicenter study. Pediatrics 93:730-736.
87. van den Anker JN, Hop WC, de Groot R, van der Heijden BJ, Broerse HM, et al. (1994) Effects of prenatal exposure to betamethasone and indomethacin on the glomerular filtration rate in the preterm infant. Pediatr Res 36: 578-581.
88. Celsi G, Kistner A, Aizman R, Eklöf AC, Ceccatelli S, et al. (1998) Prenatal dexamethasone causes oligonephronia, sodium retention, and higher blood pressure in the offspring. Pediatr Res 44: 317-322.
89. Ortiz LA, Quan A, Weinberg A, Baum M (2001) Effect of prenatal dexamethasone on rat renal development. Kidney Int 59: 1663-1669.
90. Ortiz LA, Quan A, Zarzar F, Weinberg A, Baum M (2003) Prenatal dexamethasone programs hypertension and renal injury in the rat. Hypertension 41: 328-334.
91. Stonestreet BS, Hansen NB, Laptook AR, Oh W (1983) Glucocorticoid accelerates renal functional maturation in fetal lambs. Early Hum Dev 8: 331-341.
92. Ervin MG, Berry LM, Ikegami M, Jobe AH, Padbury JF, et al. (1996) Single dose fetal betamethasone administration stabilizes postnatal glomerular filtration rate and alters endocrine function in premature lambs. Pediatr Res 40: 645-651.
93. Wintour EM, Moritz KM, Johnson K, Ricardo S, Samuel CS, et al. (2003) Reduced nephron number in adult sheep, hypertensive as a result of prenatal glucocorticoid treatment. J Physiol 549: 929-935.
94. Ervin MG, Seidner SR, Leland MM, Ikegami M, Jobe AH (1998) Direct fetal glucocorticoid treatment alters postnatal adaptation in premature newborn baboons. Am J Physiol 274: R1169-1176.
95. Moritz KM, De Matteo R, Dodic M, Jefferies AJ, Arena D, et al. (2011) Prenatal glucocorticoid exposure in the sheep alters renal development in utero: Implications for adult renal function and blood pressure control. Am J Physiol Regul Integr Comp Physiol 301: R500-509.
96. de Vries WB, van den Borne P, Goldschmeding R, de Weger RA, Bal MP, et al. (2010) Neonatal dexamethasone treatment in the rat leads to kidney damage in adulthood. Pediatr Res 67: 72-76.
97. Jahnukainen T, Chen M, Berg U, Celsi G (2001) Antenatal glucocorticoids and renal function after birth. Semin Neonatol 6: 351-355.
98. Pacifici GM (2006) Placental transfer of antibiotics administered to the mother: a review. Int J Clin Pharmacol Ther 44: 57-63.
99. Gilbert T, Cibert C, Moreau E, Géraud G, Merlet-Bénichou C (1996) Early defect in branching morphogenesis of the ureteric bud in induced nephron deficit. Kidney Int 50: 783-795.
100. Rodriguez-Barbero A, López-Novoa JM, Arévalo M (1997) Involvement of platelet-activating factor in gentamicin nephrotoxicity in rats. Exp Nephrol 5: 47-54.
101. Giapros VI, Andronikou SK, Cholevas VI, Papadopoulou ZL (2003) Renal function and effect of aminoglycoside therapy during the first ten days of life. Pediatr Nephrol 18: 46-52.
102. Kent AL, Maxwell LE, Koina ME, Falk MC, Willenborg D, et al. (2007) Renal glomeruli and tubular injury following indomethacin, ibuprofen, and gentamicin exposure in a neonatal rat model. Pediatr Res 62: 307-312.
103. Gilbert T, Lelievre-Pegorier M, Merlet-Benichou C (1990) Immediate and long-term renal effects of fetal exposure to gentamicin. Pediatr Nephrol 4: 445-450.
104. Kurki T, Eronen M, Lumme R, Ylikorkala O (1991) A randomized double-dummy comparison between indomethacin and nylidrin in threatened preterm labor. Obstet Gynecol 78: 1093-1097.
105. Pomeranz A, Korzets Z, Dolfin Z, Eliakim A, Bernheim J, et al. (1996) Acute renal failure in the neonate induced by the administration of indomethacin as a tocolytic agent. Nephrol Dial Transplant 11: 1139-1141.
106. Kent AL, Douglas-Denton R, Shadbolt B, Dahlstrom JE, Maxwell LE, et al. (2009) Indomethacin, ibuprofen and gentamicin administered during late stages of glomerulogenesis do not reduce glomerular number at 14 days of age in the neonatal rat. Pediatric Nephrology 24: 1143-1149.
107. Vanderheyden T, Kumar S, Fisk NM (2003) Fetal renal impairment. Semin Neonatol 8: 279-289.
108. Nyberg DA, Mc Gahan JP, Pretorius DH, Pilu G (2002) Diagnostic imaging of fetal anomalies. Lippincott Williams & Wilkins, Philadelphia.
109. Klaassen I, Neuhaus TJ, Mueller-Wiefel DE, Kemper MJ (2007) Antenatal oligohydramnios of renal origin: long-term outcome. Nephrol Dial Transplant 22: 432-439.
110. Casey BM, McIntire DD, Bloom SL, Lucas MJ, Santos R, et al. (2000) Pregnancy outcomes after antepartum diagnosis of oligohydramnios at or beyond 34 weeks' gestation. Am J Obstet Gynecol 182: 909-912.
111. Hinchliffe SA, Lynch MR, Sargent PH, Howard CV, Van Velzen D (1992) The effect of intrauterine growth retardation on the development of renal nephrons. Br J Obstet Gynaecol 99: 296-301.
112. Mañalich R, Reyes L, Herrera M, Melendi C, Fundora I (2000) Relationship between weight at birth and the number and size of renal glomeruli in humans: a histomorphometric study. Kidney Int 58: 770-773.
113. Fiscella K1 (1996) Racial disparities in preterm births. The role of urogenital infections. Public Health Rep 111: 104-113.
114. Schieve LA, Handler A (1996) Preterm delivery and perinatal death among black and white infants in a Chicago-area perinatal registry. Obstet Gynecol 88: 356-363.
115. de Onis M, Blössner M, Villar J (1998) Levels and patterns of intrauterine growth retardation in developing countries. Eur J Clin Nutr 52 Suppl 1: S5-15.
116. Painter RC, Roseboom TJ, Bleker OP (2005) Prenatal exposure to the Dutch famine and disease in later life: an overview. Reprod Toxicol 20: 345-352.
117. Godfrey K, Robinson S, Barker DJ, Osmond C, Cox V (1996) Maternal nutrition in early and late pregnancy in relation to placental and fetal growth. BMJ 312: 410-414.
118. Langley SC, Jackson AA (1994) Increased systolic blood pressure in adult rats induced by fetal exposure to maternal low protein diets. Clin Sci (Lond) 86: 217-222.
119. Merlet-Bénichou C, Gilbert T, Muffat-Joly M, Lelièvre-Pégorier M, Leroy B (1994) Intrauterine growth retardation leads to a permanent nephron deficit in the rat. Pediatr Nephrol 8: 175-180.
120. Desai M, Crowther NJ, Lucas A, Hales CN (1996) Organ-selective growth in the offspring of protein-restricted mothers. Br J Nutr 76: 591-603.
121. Zimanyi MA, Denton KM, Forbes JM, Thallas-Bonke V, Thomas MC, et al. (2006) A developmental nephron deficit in rats is associated with

increased susceptibility to a secondary renal injury due to advanced glycation end-products. Diabetologia 49: 801-810.

122. Lazzaroni F, Bonassi S, Magnani M, Calvi A, Repetto E, et al. (1993) Moderate maternal drinking and outcome of pregnancy. Eur J Epidemiol 9: 599-606.
123. Burguet A, Kaminski M, Abraham-Lerat L, Schaal JP, Cambonie G, et al. (2004) The complex relationship between smoking in pregnancy and very preterm delivery. Results of the Epipage study. BJOG 111: 258-265.
124. Sokol RJ, Janisse JJ, Louis JM, Bailey BN, Ager J, et al. (2007) Extreme prematurity: an alcohol-related birth effect. Alcohol Clin Exp Res 31: 1031-1037.
125. Jaddoe VW, de Ridder MA, van den Elzen AP, Hofman A, Uiterwaal CS, et al. (2008) Maternal smoking in pregnancy is associated with cholesterol development in the offspring: A 27-years follow-up study. Atherosclerosis 196: 42-48.
126. O'Leary CM, Nassar N, Kurinczuk JJ, Bower C (2009) The effect of maternal alcohol consumption on fetal growth and preterm birth. BJOG 116: 390-400.
127. Maccani MA, Avissar-Whiting M, Banister CE, McGonnigal B, Padbury JF, et al. (2010) Maternal cigarette smoking during pregnancy is associated with downregulation of miR-16, miR-, and miR-146a in the placenta. Epigenetics 5: 583-589.
128. Dejmek J, Solansk y I, Podrazilova K, Sram RJ (2002) The exposure of nonsmoking and smoking mothers to environmental tobacco smoke during different gestational phases and fetal growth. Environ Health Perspect.110:601-606.
129. Henriksen T, Clausen T (2002) The fetal origins hypothesis: placental insufficiency and inheritance versus maternal malnutrition in well-nourished populations. Acta Obstet Gynecol Scand 81: 112-114.
130. Schreuder M, Delemarre-van de Waal H, van Wijk A (2006) Consequences of intrauterine growth restriction for the kidney. Kidney Blood Press Res 29: 108-125.
131. Baschat AA, Cosmi E, Bilardo CM, Wolf H, Berg C, et al. (2007) Predictors of neonatal outcome in early-onset placental dysfunction. Obstet Gynecol 109: 253-261.
132. Ananth CV, Smulian JC, Srinivas N, Getahun D, Salihu HM (2005) Risk of infant mortality among twins in relation to placental abruption: contributions of preterm birth and restricted fetal growth. Twin Res Hum Genet 8: 524-531.
133. Lyell DJ, Lambert-Messerlian GM, Giudice LC (2003) Prenatal screening, epidemiology, diagnosis, and management of preeclampsia. Clin Lab Med 23: 413-442.
134. Duley L (2009) The global impact of pre-eclampsia and eclampsia. Semin Perinatol 33: 130-137.
135. Sibai B, Dekker G, Kupferminc M (2005) Pre-eclampsia. Lancet 365: 785-799.
136. Churchill D, Perry IJ, Beevers DG (1997) Ambulatory blood pressure in pregnancy and fetal growth. Lancet 349: 7-10.
137. Walker BR, McConnachie A, Noon JP, Webb DJ, Watt GC (1998) Contribution of parental blood pressures to association between low birth weight and adult high blood pressure: cross sectional study. BMJ 316: 834-837.
138. Sibai BM (2003) Diagnosis and management of gestational hypertension and preeclampsia. Obstet Gynecol 102: 181-192.
139. López-Jaramillo P, García RG, López M (2005) Preventing pregnancy-induced hypertension: are there regional differences for this global problem? J Hypertens 23: 1121-1129.
140. Smith AP, Ong S, Smith NC, Campbell D (2001) A prospective longitudinal study of growth velocity in twin pregnancy. Ultrasound Obstet Gynecol 18: 485-487.
141. Keith L, Oleszczuk JJ (1999) Iatrogenic multiple birth, multiple pregnancy and assisted reproductive technologies. Int J Gynaecol Obstet 64: 11-25.
142. Fauser BC, Devroey P, Macklon NS (2005) Multiple birth resulting from ovarian stimulation for subfertility treatment. Lancet 365: 1807-1816.
143. Lynch A, McDuffie R, Stephens J, Murphy J, Faber K, et al. (2003) The contribution of assisted conception, chorionicity and other risk factors to very low birthweight in a twin cohort. BJOG 110: 405-410.
144. Chang YL, Chang SD, Chao AS, Wang CN, Wang TH, et al. (2011) The relationships of umbilical venous volume flow, birthweight and placental share in monochorionic twin pregnancies with and without selective intrauterine growth restriction. Twin Res Hum Genet 14: 192-197.
145. Breathnach FM, Malone FD (2012) Fetal growth disorders in twin gestations. Semin Perinatol 36: 175-181.
146. Lucas SR, Costa Silva VL, Miraglia SM, Zaladek Gil F (1997) Functional and morphometric evaluation of offspring kidney after intrauterine undernutrition. Pediatr Nephrol 11: 719-723.
147. Louey S, Cock ML, Stevenson KM, Harding R (2000) Placental insufficiency and fetal growth restriction lead to postnatal hypotension and altered postnatal growth in sheep. Pediatr Res 48: 808-814.
148. Ozaki T, Nishina H, Hanson MA, Poston L (2001) Dietary restriction in pregnant rats causes gender-related hypertension and vascular dysfunction in offspring. J Physiol 530: 141-152.
149. Mitchell EK, Louey S, Cock ML, Harding R, Black MJ (2004) Nephron endowment and filtration surface area in the kidney after growth restriction of fetal sheep. Pediatr Res 55: 769-773.
150. Moritz KM, Mazzuca MQ, Siebel AL, Mibus A, Arena D, et al. (2009) Uteroplacental insufficiency causes a nephron deficit, modest renal insufficiency but no hypertension with ageing in female rats. J Physiol 587: 2635-2646.
151. Zohdi V, Moritz KM, Bubb KJ, Cock ML, Wreford N, et al. (2007) Nephrogenesis and the renal renin-angiotensin system in fetal sheep: effects of intrauterine growth restriction during late gestation. Am J Physiol Regul Integr Comp Physiol 293: R1267-1273.
152. Sutherland MR, Gubhaju L, Yoder BA, Stahlman MT, Black MJ (2009) The effects of postnatal retinoic acid administration on nephron endowment in the preterm baboon kidney. Pediatr Res 65: 397-402.
153. Holland P (1993) Placental insufficiency and its effect on the fetus and adult disease. Lancet 341: 827.
154. Veille JC, McNeil S, Hanson R, Smith N (1998) Renal Hemodynamics: Longitudinal Study from the Late Fetal Life to One Year of Age J Matern Fetal Investig 8: 6-10.
155. Arant BS Jr (1987) Postnatal development of renal function during the first year of life. Pediatr Nephrol 1: 308-313.
156. Tulassay T, Vásárhelyi B (2002) Birth weight and renal function. Curr Opin Nephrol Hypertens 11: 347-352.
157. Rodesch F, Simon P, Donner C, Jauniaux E (1992) Oxygen measurements in endometrial and trophoblastic tissues during early pregnancy. Obstet Gynecol 80: 283-285.
158. Fischer B, Bavister BD (1993) Oxygen tension in the oviduct and uterus of rhesus monkeys, hamsters and rabbits. J Reprod Fertil 99: 673-679.
159. Tufro-McReddie A, Norwood VF, Aylor KW, Botkin SJ, Carey RM, et al. (1997) Oxygen regulates vascular endothelial growth factor-mediated vasculogenesis and tubulogenesis. Dev Biol 183: 139-149.
160. Finer N, Saugstad O, Vento M, Barrington K, Davis P, et al. (2010) Use of oxygen for resuscitation of the extremely low birth weight infant. Pediatrics 125: 389-391.
161. Kamlin CO, O'Donnell CP, Davis PG, Morley CJ (2006) Oxygen saturation in healthy infants immediately after birth. J Pediatr 148: 585-589.
162. AHA, AAP (2006) 2005 American heart association (aha) guidelines for cardiopulmonary resuscitation (cpr) and emergency cardiovascular care (ecc) of pediatric and neonatal patients: Neonatal resuscitation guidelines. Pediatrics 117: e1029-1038.
163. Tan A, Schulze A, O'Donnell CP, Davis PG (2005) Air versus oxygen for resuscitation of infants at birth. Cochrane Database Syst Rev 18: CD002273.
164. Rabi Y, Singhal N, Nettel-Aguirre A (2011) Room-air versus oxygen administration for resuscitation of preterm infants: the ROAR study. Pediatrics 128: e374-381.

165. Vento M, Asensi M, Sastre J, Lloret A, García-Sala F, et al. (2003) Oxidative stress in asphyxiated term infants resuscitated with 100% oxygen. J Pediatr 142: 240-246.

166. Winterbourn CC (2008) Reconciling the chemistry and biology of reactive oxygen species. Nat Chem Biol 4: 278-286.

167. Saugstad OD (2001) Update on oxygen radical disease in neonatology. Curr Opin Obstet Gynecol 13: 147-153.

168. Perrone S, Mussap M, Longini M, Fanos V, Bellieni CV, et al. (2007) Oxidative kidney damage in preterm newborns during perinatal period. Clin Biochem 40: 656-660.

169. Yzydorczyk C, Comte B, Cambonie G, Lavoie JC, Germain N, et al. (2008) Neonatal oxygen exposure in rats leads to cardiovascular and renal alterations in adulthood. Hypertension 52: 889-895.

170. Sutherland MR, O'Reilly M, Kenna K, Ong K, Harding R, et al. (2013) Neonatal hyperoxia: effects on nephrogenesis and long-term glomerular structure. Am J Physiol Renal Physiol 304: F1308-1316.

171. Gilbert T, Gaonach S, Moreau E, Merlet-Benichou C (1994) Defect of nephrogenesis induced by gentamicin in rat metanephric organ culture. Lab Invest 70: 656-666.

172. Nathanson S, Moreau E, Merlet-Benichou C, Gilbert T (2000) In utero and in vitro exposure to beta-lactams impair kidney development in the rat. J Am Soc Nephrol 11: 874-884.

173. Cullen L, Young R, Bertram J (2000) Studies on the effects of gentamicin on rat metanephric development in vitro. Nephrology. 5: 115-123.

174. Nagai J, Takano M (2004) Molecular aspects of renal handling of aminoglycosides and strategies for preventing the nephrotoxicity. Drug Metab Pharmacokinet 19: 159-170.

175. Kent AL, Brown L, Broom M, Broomfield A, Dahlstrom JE (2012) Increased urinary podocytes following indomethacin suggests drug-induced glomerular injury. Pediatr Nephrol 27: 1111-1117.

176. Allegaert K (2009) The impact of ibuprofen or indomethacin on renal drug clearance in neonates. J Matern Fetal Neonatal Med 22 Suppl 3: 88-91.

177. Olliges A, Wimmer S, Nüsing RM (2011) Defects in mouse nephrogenesis induced by selective and non-selective cyclooxygenase-2 inhibitors. Br J Pharmacol 163: 927-936.

178. Sutherland MR, Yoder BA, McCurnin D, Seidner S, Gubhaju L, et al. (2012) Effects of ibuprofen treatment on the developing preterm baboon kidney. Am J Physiol Renal Physiol 302: F1286-1292.

179. Langhendries JP, Battisti O, Bertrand JM (1988) Aminoglycoside nephrotoxicity and urinary excretion of N-acetyl-beta-D-glucosaminidase. Biol Neonate 53: 253-259.

180. Tugay S, Bircan Z, CaÄŸlayan C, Arisoy AE, Gkalp AS (2006) Acute effects of gentamicin on glomerular and tubular functions in preterm neonates. Pediatr Nephrol 21: 1389-1392.

181. Clark RH, Thomas P, Peabody J (2003) Extrauterine growth restriction remains a serious problem in prematurely born neonates. Pediatrics 111: 986-990.

182. Bacchetta J, Harambat J, Dubourg L, Guy B, Liutkus A, et al. (2009) Both extrauterine and intrauterine growth restriction impair renal function in children born very preterm. Kidney Int 76: 445-452.

183. Schmidt IM, Damgaard IN, Boisen KA, Mau C, Chellakooty M, et al. (2004) Increased kidney growth in formula-fed versus breast-fed healthy infants. Pediatr Nephrol 19: 1137-1144.

Chapter Two:
LATE GESTATIONAL DEVELOPMENT OF THE HUMAN FETAL KIDNEY: WIDE VARIATION IN THE TIMING OF THE CESSATION OF NEPHROGENESIS

CHAPTER TWO DECLARATION

Declaration by candidate

[It is to be noted that I have used my married surname (Ryan) in this manuscript]

Chapter 2 has been submitted on the 12th of August 2016 to the Journal of American Society of Nephrology and is currently under review. Reprinted in this thesis is a copy of the submitted manuscript.

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

Nature of contribution	Extent of contribution (%)
I conducted the majority of the experiments. Performed all statistical analyses. Wrote the manuscript.	80%

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

Name	Nature of contribution	Extent of contribution (%) for student co-authors only	Co-author(s), Monash student Y/N
Tracey Flores Alison Kent, Jane Dahlstrom, Victor Puelles, John Bertram, Andrew McMahon, Melissa Little, Lynette Moore, M. Jane Black	Involved in the collection of tissue at autopsy, provided medical and autopsy reports, involved in the design of the experiments, obtained funding, assisted with some experimental work		N

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

Candidate's Signature	[redacted]	Date 21.9.16
Main Supervisor's Signature		Date 21.9.16

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LATE GESTATIONAL DEVELOPMENT OF THE HUMAN FETAL KIDNEY: WIDE VARIATION IN THE TIMING OF THE CESSATION OF NEPHROGENESIS

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Abstract

During normal human development, nephrogenesis (the formation of nephrons) is complete by birth, with the majority of nephrons formed late in gestation. The aims of this study were to morphologically examine nephrogenesis and glomerulogenesis in fetal human kidneys from 20 to 41 weeks of gestation. The kidneys from female and male infants were analysed separately. Kidney samples were obtained at autopsy from 81 infants that died acutely *in utero* or died within 24 hours after birth. Using image analysis, nephrogenic zone width, the number of glomerular generations, renal corpuscle cross-sectional area and the cellular composition of glomeruli were examined. The number of glomerular generations formed within the kidney was directly proportional to gestational age, body weight and kidney weight, with variability in the ultimate number of glomerular generations formed within the kidneys (8 to 12 generations) and in the timing of the cessation of nephrogenesis (as early as 33 weeks in one infant and still ongoing at 37 weeks in another infant). There was a small but significant increase in renal corpuscle cross-sectional area from mid gestation to term in females, but this was not evident in males. The proportion of podocytes, endothelial and non-epithelial cells within glomeruli did not differ in kidneys at mid gestation, late gestation and term. In conclusion, the findings highlight spatial and temporal variability in nephrogenesis in the developing human kidney, whereas the relative cellular composition of glomeruli does not appear to be influenced by gestational age or glomerular size.

Introduction

Nephrogenesis (the formation of nephrons) occurs in early development and is complete by birth in the term born human infant. [1-3] Initially the development of the permanent kidney (the metanephros) commences in weeks 4-5 of gestation when the ureteric bud first branches from the Wolffian duct into the metanephric mesenchyme.[4] Subsequent inductive interactions of the mesenteric mesenchyme and the bud tips lead to nephron formation, [5] with the first nephrons formed by 8-9 weeks of gestation.[2, 3] Up until 15 weeks of gestation there is dichotomous branching of the ureteric duct which ultimately leads to formation of the renal calyx and to the first generations of nephrons.[3, 4] After this time, nephrons form via cascade formation from the terminal branch tips and subsequently, additional generations of nephrons are formed beyond the zone of growth and attach directly to the collecting duct.[3, 4] The majority of nephrons (approximately 60%) are formed in the second half of gestation.[6]

Studies of autopsied kidneys have shown that there is a wide range in nephron number in the normal human kidney; ranging from approximately 250,000 to over 2 million nephrons per kidney.[7-10] The mechanisms leading to such a wide variation in nephron endowment are currently unknown. Indeed, genetic variability, differences in the *in utero* environment, as well as exposure to postnatal renal insults throughout life are likely key factors in the variability of nephron endowment in adulthood.[11-14] Since loss of glomeruli ultimately leads to renal disease[15, 16] it is likely that individuals that are born with a high nephron endowment will be relatively protected from renal disease later in life, whereas individuals born with a low nephron endowment are likely to be more vulnerable to postnatal renal insults. Hence, in order to preserve long-term renal health

it is imperative to maximise nephron endowment at birth. In order to develop strategies to do this, it is essential to develop an understanding of how factors during pregnancy influence the developing kidney. Over recent years valuable knowledge relating to the regulation of nephrogenesis has been derived from animal models (predominantly rodent models);[17] however, whether these findings can be fully extrapolated to the developing human infant is equivocal, given that the temporal and spatial development of nephrogenesis is quite different in rodents compared to the human.[3] For example, unlike the human, in rodents the kidneys have only one papilla and branching morphogenesis of the ureteric duct is the key mediator of nephron endowment, with the majority of nephrons formed after birth.[3, 18] Apart from anatomical microdissection studies, conducted many decades ago, describing the process of nephrogenesis in the human kidney,[4, 5, 19, 20] there have been very few studies of nephrogenesis in the fetal human kidney (and these have mainly been conducted in only a small number of infants).[6, 21-24]

To further enhance our knowledge of the normal development of the human kidney, in this study we conducted a comprehensive histological examination of the developing human kidney from 20 weeks in gestation until term; the developmental period when the majority of nephrons are formed. The aims were to examine: nephrogenic zone width, the number of glomerular generations, glomerular size and the proportion of the different cell types within the glomerulus, as well as to explore the variability in the timing in the cessation of nephrogenesis. The kidneys from male and female infants were analysed separately in order to examine whether there were sex differences in renal

development during this period. Only the kidneys from infants that were normally grown and died acutely *in utero* were analysed.

Results

Body weight gain over late gestational development

Body weights of infants ranging in age from 20 to 41 weeks of gestation are shown in (Figure 1). The body weights represent the weights of female and male infants at delivery (n = 66: 29 females; and 37 males). Given that body weight is affected by the period of time to autopsy we have only presented the body weights at the time of birth. Birth weights were not available for all infants. As expected, from 20 weeks of gestation until term there was a strong linear correlation between body weight and gestational age in both male and female infants (Female r = 0.960, $p < 0.0001$; Male: r = 0.956, $p < 0.0001$). Overall, female infants were significantly lighter (P elevation = 0.014) than male infants, however, the weight gain over the gestational period was not different (P slope = 0.863) between males and females. Similar trends were observed for autopsied weights over gestational age (data not shown).

Kidney weight gain over late gestational development

Similar to body growth weight gain over time, there was a significant linear correlation between kidney weight and gestational age over the study period in both male and female infants (Female r = 0.887, $p <0.0001$; Male: r = 0.911, $P < 0.0001$) (Figure 2A). In accordance with body weight, there was a trend for kidney weights to be lighter in female infants, but this did not quite reach statistical significance (P = 0.059); the gain in kidney weight over gestation was not different between males and females. Overall kidney weight over time was directly proportional to body weight over time in both females and males throughout gestation; hence kidney weight adjusted for body weight, remained constant over the gestational period (Figure 2B).

Assessment of Nephrogenesis

Nephrogenic zone width

Of the 81 kidneys studied (from 81 infants), nephrogenesis was ongoing in 20 of the 43 females and 26 of the 38 males at the time of autopsy; with evidence of metanephric mesenchyme and developing glomeruli in the form of comma and S-shaped bodies in the outer renal cortex. In both female and male infants, nephrogenic zone width decreased with increasing gestational age (Figure 3), with a significant inverse correlation between nephrogenic zone width and gestational age (Female $r = 0.828$, $P < 0.0001$; Male: $r = 0.906$; $P < 0.0001$). In 10 infants (8 females and 2 males) born prior to 38 weeks of gestation there was no evidence of a nephrogenic zone, thus indicating that nephrogenesis was complete in these kidneys at the time of autopsy. There was no difference in nephrogenic zone width or change in nephrogenic zone width over the gestational period between females and males.

Timing of the cessation of nephrogenesis

Table 1 indicates whether nephrogenesis was ongoing or had ceased at the time of autopsy in all kidneys studied. Nephrogenesis was ongoing in the kidneys of all infants ranging in age from 20 to 31 weeks of gestation. Interestingly, from 33 weeks of gestation there was a wide range in the timing of the cessation of nephrogenesis. Figure 4 shows kidneys from two infants at the same gestational age with nephrogenesis ongoing in one of the kidneys and complete in the other. Nephrogenesis had ceased in a female infant as early as 33 weeks' gestation and in a male infant nephrogenesis was still ongoing at 37 weeks of gestation. In all term kidneys (27 term kidneys: 15 females and 13 males), nephrogenesis was complete at the time of autopsy. Overall, from 35 weeks in gestation, nephrogenesis was complete in the majority of the kidneys examined; at 35 weeks of gestation, nephrogenesis was complete in 2 out of 3 kidneys; at 36 weeks of gestation

nephrogenesis was complete in 3 out of 4 kidneys and at 37 weeks of gestation nephrogenesis was complete in 4 out of 5 kidneys.

The number of glomerular generations

There was a strong linear relationship between the number of glomerular generations with increasing gestational age in both female and male infants from 20 weeks of gestation until term (Female; $r = 0.901$; $P < 0.0001$; Male $r = 0.9099$; $P < 0.0001$) (Figure 5A). Likewise, there was a strong relationship between the number of glomerular generations with kidney weight (Female: $r = 0.799$, $P < 0.0001$; Male: $r = 0.879$, $P < 0.0001$) (Figure 5B) and body weight (Female: $r = 0.804$, $P < 0.0001$; Male: $r = 0.868$; $P < 0.0001$) (Figure 5C). There was no significant difference between the sexes in the number of glomerular generations formed over the gestational period ($P=0.69$). In the kidneys where nephrogenesis was complete, the average number of glomerular generations was not significantly different between the sexes, with an average of 10 glomerular generations formed in both male and female kidneys. Notably, however, in the infants where nephrogenesis had ceased at the time of autopsy (ranging in age from 33 to 41 weeks' gestation), the number of glomerular generations varied; ranging from 8 to 12 glomerular generations per kidney.

Renal corpuscle cross-sectional area

Female and male infants demonstrated differences in the growth of glomeruli with increasing gestational age (Figure 6A). In the female kidneys, from 20 weeks of gestation until term, there was a small but significant association between renal corpuscle cross-sectional area and gestational age ($r = 0.561$, $P = 0.001$), whereby renal corpuscle cross-sectional area increased with increasing gestational age. Interestingly, in the male kidneys this relationship between renal corpuscle cross-sectional area and gestational age was not evident (Male: $r = 0.0643$, $P = 0.7013$). In addition, in the female kidneys, there was a significant linear relationship between renal

corpuscle cross-sectional area relative to kidney weight (r = 0.668, P < 0.0001) (Figure 7B) and renal corpuscle cross-sectional area relative to body weight (r = 0.560, P < 0.0001) (Figure 7C). In males, however, associations between renal corpuscle cross-sectional area and kidney weight (r = 0.308, P = 0.0601) (Figure 6B) and body weight (r = 0.264, P = 0.1321) (Figure 6C) were not evident. Additionally, only the female kidneys demonstrated an association over the gestational period between renal corpuscle cross-sectional area and the number of glomerular generations formed within the cortex (Female: r = 0.544, P = 0.0016; Male: r = 0.172, P = 0.3241) (Figure 6D). At term, there was no difference in the average renal corpuscle cross-sectional area between males and females.

Assessment of the number of glomerular cell types

In 14 of the 81 kidneys studied (8 males and 6 females) the relative proportion of the different cell types within the glomeruli were examined.

The average number of podocytes, endothelial cells and non-epithelial cells per glomerular cross-section in fully formed glomeruli for each kidney studied over the gestational period are shown in Figures 7A (male infants) and 7B (Female infants). In the fully formed glomeruli, the average number of glomerular cell types per glomerular cross-section, remained constant across gestational development with no apparent relationship between the average number of podocytes, endothelial cells or non-epithelial cells with gestational age. Overall, the average proportion of podocytes within the glomeruli was 64.4 ± 0.9%, the proportion of the endothelial cells was 27.6 ± 1.0 % and non-epithelial cells was 8.0 ± 0.5 %. It is expected that the majority of the non-epithelial cells would be resident mesangial cells; however, this could not be verified.

Discussion

This comprehensive study conducted in human kidneys shows that kidney growth in the latter half of gestation is directly proportional to body weight and gestational age in both

male and female infants. Overall, female infants were lighter and had a reduced kidney weight relative to their age-matched male counterparts; however, their body and kidney weight gain over time throughout the gestational period was not different to males. Importantly, the findings demonstrate wide variation in the timing of the cessation of nephrogenesis and in the number of glomerular generations formed within the kidneys. Interestingly, there was a significant 28% increase in glomerular size from mid gestation to term, (as determined by measurement of renal corpuscle cross-sectional area), in female infants, whereas glomerular size remained relatively constant over the gestational period in male infants. Overall, the number and proportion of podocytes, endothelial and non-epithelial cells per glomerular cross-section did not differ in kidneys at mid gestation, late gestation and term.

To date there have been relatively few studies that have examined nephrogenesis during late gestational development in the human kidney.[6, 21-24] This is likely due to the fact that such analyses can only be undertaken in the kidneys of deceased infants. The majority of the previous studies have analysed only a small cohort of infants and the data is often confounded by many of the infants being intrauterine growth restricted prior to birth. This is an important limitation because it is well established in animal models that intrauterine growth restriction adversely impacts nephrogenesis leading to a reduction in nephron endowment.[1, 25] Hence, in the present study we have carefully selected the kidneys to be studied. All infants that were intrauterine growth restricted or exposed to chorioamnionitis (which can also adversely impact nephrogenesis) were excluded from the study. [26] Importantly, we were also careful to only analyse kidneys, with minimal tissue maceration, that were obtained from infants that had been growing normally and

died suddenly *in utero;* this information was derived from the autopsy reports. Using this approach, we eliminated infants that may have been in poor health over a chronic period *in utero*, prior to their demise, which may have affected renal development.

Males are larger than age-matched females with kidney weight proportional to body weight

In the present study, as expected body weight was strongly correlated to gestational age in both female and male infants. Female infants were significantly lighter in weight overall, however relative weight gain between the sexes was similar over the gestational period. Our findings are consistent with previous studies,[27, 28] reporting that male infants in late gestation are heavier at birth than females at the same gestational age. In accordance with body weights, there was a strong linear correlation between kidney weight and gestational age in both male and female infants, As a result the kidney weight to body weight ratio remained constant over the gestational period; similar findings in relation to kidney growth (length and volume measured using prenatal ultrasonography) are reported in the literature. [29-33] Overall, kidney weights in female infants were less than in male infants. Although this did not quite reach statistical significance in the present study (P = 0.059), there are a number of reports in the literature showing that kidney size during development (length and volume) is less in females than in males. [28, 29] Notably our findings show that although kidney weights are less in females than males, the growth of the kidneys (weight gain over time) in the second half of gestation was not different between the sexes.

Variation in the timing of the cessation of nephrogenesis and in the number of glomerular generations formed

As expected, in this study there was a strong negative correlation between the width of the nephrogenic zone and gestational age. In both female and male infants, at 20 weeks of gestation there was a wide nephrogenic zone indicative of active nephrogenesis with an average width of around 200 µm; this was halved by 30 weeks gestation and by term it was no longer apparent. Importantly, the findings of this study clearly show that there is considerable variation in the cessation of the timing of nephrogenesis in the human kidney, which until now has not been well recognised. Nephrogenesis is often quoted in the literature to be completed at around 36 weeks' gestation. This is somewhat misleading, in that nephrogenesis ceases much earlier in some kidneys. In the present study, nephrogenesis was complete as early as 33 weeks' gestation in one of the kidneys examined and in a previous study we have observed nephrogenesis to be complete even earlier in a human kidney, at 32 weeks of gestation. [23]

In this study, we were unable to directly determine the number of glomeruli/nephrons within the kidneys as it was not known what fraction of the whole kidney was collected, fixed and embedded at autopsy. As a surrogate indicator of the number of nephrons formed within the kidney, we examined the number of glomerular generations formed within the renal cortex; given that the number of glomerular generations correlates with the number of nephrons formed within the kidney.[34] Interestingly, we found that early cessation of nephrogenesis did not necessarily result in fewer generations of glomeruli formed within the kidney. In the kidney where nephrogenesis was completed early at 33 weeks' gestation there were 11 glomerular generations and in kidneys where nephrogenesis was ongoing later in gestation there was variability in the number of glomerular generations formed; ranging from 10 to 12 glomerular generations. Hence,

although it remains likely that earlier cessation of nephrogenesis will impact the number of nephrons formed in the developing fetal kidney, this appears to not necessarily be the case.

How final nephron number is determined in the mammalian kidney is not known. [35] In rodents, where there is evidence for nephron formation in the immediate postnatal period,[18, 36] cessation of this process could have been explained by an extrinsic environmental trigger associated with parturition or an intrinsic clock triggering differentiation. [36, 37] Recent studies in the mouse showed that a nephron progenitor cell from late in development, when transplanted back into an earlier niche, can continue to proliferate as a progenitor without committing to nephron formation if surrounded by younger progenitors.[38] However, there was a clear distinction in gene expression and proliferation rate between nephron progenitors isolated early versus late in gestation, supporting an intrinsic model of aging. [38] In the human, a parturition-based trigger appeared unlikely given the assumption that nephron formation was complete some weeks before birth. Here we report considerable variation in the timing of cessation of nephrogenesis in the human kidney. This observed variability does not support an extrinsic regulation of this process. Importantly, in those instances with the earliest evidence of cessation of nephrogenesis, there was no apparent change in the number of glomerular generations, suggesting that these kidneys have reached their potential faster. This also suggests an intrinsic mechanism for determining total nephron number but reveals that the same end result may be reached at different rates. Understanding what controls the pace at which a final complement is attained will be important for evaluating risk and improving outcomes.

Differences in the growth of glomeruli between the sexes

An interesting finding in this study was the apparent sexual dimorphism in the size of glomeruli over the gestational period. In the female infants there was a small but positive linear relationship between renal corpuscle cross-sectional area and gestational age, (as well as body and kidney weight); average renal corpuscle cross-sectional area was 3631 ± 594 μm^2 at 23 weeks of gestation and was 5058 ± 268 μm^2 at term. To the contrary, in males renal corpuscle size remained relatively constant over the gestational period with renal corpuscle cross-sectional area averaging 4480 ± 267 μm^2 at 23 weeks of gestation and 4839 ± 157 μm^2 at term; there was no apparent association between renal corpuscle cross-sectional area and gestational age, body weight or kidney weight. It is important to keep in mind when interpreting findings associated with glomerular cross-sectional area, that the glomeruli when sampled would not have all been cut in the central plane; however, with 100 glomeruli sampled per kidney it is expected that the average value should be a relatively accurate measure of glomerular cross-sectional area.

Why there appears to be sexual dimorphism in glomerular growth in the second half of gestation is currently unknown. Indeed, it is an interesting finding of this study and warrants further investigation.

Proportion of podocytes within glomeruli remains constant

Podocytes play a critical role in glomerular filtration. An important aspect of our study was to examine the cellular composition of glomeruli over the gestational study period and in particular the growth of podocytes. We were restricted in the number of kidneys we could analyse, by whether or not the immunostaining was successful; this was likely

dependent on the lag time between death and autopsy, when the kidney tissue was collected and fixed.

Importantly our findings suggest that once a glomerulus has formed, whether it is early in gestation or late in gestation, the proportion of podocytes relative to other cell types within the glomerulus appears to remain relatively constant; this was applicable to the kidneys of both male and female infants. When mature glomeruli were examined in mid-gestation the proportion of podocytes, endothelial cells and other non-epithelial cells (predominantly mesangial cells) were not different compared to mature glomeruli of kidneys from late in gestation or at term. The proportion of podocytes to endothelial cells and non-epithelial cell types remained constant at approximately 64%, 28% and 8%, respectively. A limitation of our study is that podocyte, endothelial cell and non-epithelial cell number has been assessed in glomerular cross-sections (50 per kidney) and the absolute number of podocytes per glomerulus was not determined. Such analyses would require an optical disector stereological approach [39] (a very time-consuming and tedious approach) which was considered not feasible for a study of these dimensions.

Conclusion

In conclusion, the findings highlight the considerable variation in the gestational timing of the cessation of nephrogenesis and in the number of glomerular generations formed within the kidney. Future studies are required to elucidate the mechanisms leading to the cessation of nephrogenesis and for the initiation of new glomerular generations within the developing kidney as both have the potential to impact total nephron endowment.

Methods

Study groups

In this retrospective study, archived fetal and newborn kidneys were obtained from the Women's and Children's Hospital in North Adelaide, South Australia, and the Canberra Hospital in The Australian Capital Territory. The kidneys were collected at autopsy from infants that died suddenly *in utero* or died within 24 hours after birth, ranging in age from 20-41 weeks of gestation (n = 38 female; n = 43 male). Autopsies were performed between the years 1996 and 2013. Only the kidneys from infants with written informed consent from the parents were included in the study. Infants were excluded from the study if there was evidence of congenital abnormalities, intrauterine growth restriction, cardiovascular or renal complications or exposure to chorioamnionitis *in utero.* Kidneys were also excluded if there was severe maceration of the tissue.

Ethical approval for this study 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.

Tissue preparation and processing

Kidneys collected at autopsy (one kidney per baby; in most cases the left kidney) were weighed and cut into two in the longitudinal plane; large kidneys were further cut transversely. The kidney portions were embedded in paraffin and sectioned at 5 µm. Kidney sections with a visible cortex and medulla were stained with haematoxylin and eosin for the assessment of nephrogenic zone width, number of glomerular generations and renal corpuscle area. Additionally, analyses of the glomerular cell types were

undertaken in a subset of the archived kidneys by immunofluorescence; this was restricted to kidneys where the application of immunofluorescence was possible.

Assessment of nephrogenesis

In all kidneys it was noted whether nephrogenesis was ongoing or complete. Nephrogenesis was considered ongoing if there was evidence of metanephric mesenchyme and immature nephrons in the form of comma and S-shaped bodies in the outer renal cortex.

Nephrogenic zone width

In kidneys where nephrogenesis was ongoing, the width of the nephrogenic zone was measured in four randomly sampled regions of the cortex using image analysis (Image Pro Plus v. 6.0 for Windows, Media Cybernetics; Silver Spring, MD, USA) and average nephrogenic zone width per kidney determined. [23, 34]

Number of glomerular generations

The number of glomerular generations formed within the kidneys was assessed in haematoxylin and eosin stained sections, using a medullary ray glomerular counting method. [6, 22, 23, 40] Utilising this technique, mature glomeruli were counted along 5 clearly defined medullary rays per kidney, that extended from the cortico-medullary junction to the outer renal cortex and the average number of glomerular generations per kidney determined.

Assessment of glomerular size and proportion of glomerular cell types

Renal corpuscle cross sectional area

Glomerular size was assessed in the majority of the kidneys (32 females and 38 males), by measuring the cross-sectional area of the renal corpuscle; this was considered a more accurate assessment of glomerular size rather than glomerular tuft area, given that the kidneys were not perfusion-fixed and so it was likely that the glomerular capillaries may not be patent. At least 100 renal corpuscle cross-sectional areas per kidney were measured using image analysis software (Image Pro Plus v. 6.0 for Windows, Media Cybernetics; Silver Spring, MD, USA). To do this, a complete kidney section stained with haematoxylin and eosin from each kidney was selected at random. The kidney sections were then sampled using a systematic sampling technique at X400 magnification with a step length of 1mm x 1mm. At each field of view one glomerulus was measured. If more than one glomerulus was observed in the field of view, the renal corpuscle for analysis was then selected according to the method of Nyengaard and Marcussen [41]. The cross-sectional area was determined by tracing the inner boundary of the Bowman's capsule. The average renal corpuscle cross-sectional area was then calculated for each kidney.

Assessment of glomerular cell types

Immunohistochemical identification of resident glomerular cell types

A standard immunofluorescence protocol[39, 42, 43] was used in 5 µm paraffin embedded kidney sections to identify podocytes, endothelial and non-epithelial cells within late maturing glomeruli; glomeruli in stages I, II and III of glomerular development were analysed.[21, 23] Briefly, Immunohistochemistry for glomerular cell identification was performed with an automated system, using a DAKO Autostainer Plus Staining System. Initially, sections were rehydrated and subjected to heat-induced antigen retrieval for 30 minutes at 98^{o} C in 10x citrate buffer at pH = 6.0. An antibody against Wilms' Tumour-1

(WT-1) antigen (monoclonal mouse anti-human WT-1; DAKO M356101, clone 6F-H2 [1:50]) was used for the identification of podocytes; the WT-1 antibody has been previously validated to be cytoplasmic and specific in the staining of podocytes. [39, 42, 43] An antibody against von Willebrand Factor (vWF; polyclonal rabbit anti-human vWF-DAKO A008202) [1:200]), was used for the identification of endothelial cells. All nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI; 1:10,000 – Sigma–AldrichD9542-10M6). Remaining cells within the glomeruli, with a visible nucleus but unstained cytoplasm, were classified as non-epithelial cells; the vast majority of these cells would be mesangial cells given that other cell types within the glomeruli are rare and there was no evidence of inflammatory infiltrates in the glomeruli examined.

The sections were scanned at different wavelengths and virtual images obtained at a magnification of X400 using an Aperio ScanScope AT Turbo (Leica Biosystems, Vista, California, USA); DAPI (370nm), WT-1 (488nm), vWF (555nm).

Proportion of Podocytes, endothelial and non-epithelial cells per glomerulus

Immunofluorescence was successfully achieved in 14 of the kidneys studied (n = 8 males and n = 6 females). Sub-standard immunofluorescence in the remaining kidney sections was due to inadequate fixation which was likely due to the lag time between death and autopsy (when the kidney portions were fixed). The proportions of the different glomerular cell types were examined. Using Image J (Image Pro Plus v. 6.0 for Windows, Media Cybernetics; Silver Spring, MD, USA), the absolute number of podocytes, endothelial and non-epithelial cells per glomerulus were determined in composite images (Figure 8A) of 50 glomerular cross-sections per kidney (approximately 16 from each of the outer, middle and inner cortex). Initially the glomerular nuclei were visualised

using DAPI and the total number of nuclei counted. Subsequently, positive WT-1 stained podocytes (Figure 8B) and positive vWF endothelial cells (Figure 8C) were identified and counted. Cells not labelled as podocytes or endothelial cells in the composite image were classified as non-epithelial cells. The relative proportions of each cell type was then calculated.

Statistical analysis

All data were analysed using GraphPad Prism v5.03 for windows (GraphPad Software, San Diego, California). Linear regression analyses were undertaken to determine correlations between the indices of fetal growth (birth weight and kidney weight) and renal morphology (nephrogenic zone width, glomerular generation number, glomerular cross-sectional area) versus gestational age. Male infants were examined separately to female infants with an analysis of co-variance performed to compare the regression lines. Statistical significance was accepted as $p < 0.05$.

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Statement of competing financial interests

None

References

1. Moritz, KM, Wintour, EM, Black, MJ, Bertram, JF, Caruana, G: Factors influencing mammalian kidney development: implications for health in adult life. *Adv Anat Embryol Cell Biol,* 196**:** 1-78, 2008.

2. Woolf, AS, Winyard, PJ, Hermanns, MH, Welham, SMJ: Maldevelopment of the Human Kidney and Lower Urinary Tract: An Overview. In: *The Kidney: From Normal Development to Congenital Disease.* edited by VIZE PD, W. A., BARD JBL,, Academic Press, 2003, pp 377-393.

3. Cullen-McEwen, L, Sutherland, MR, Black, MJ: Chapter 3 - The Human Kidney: Parallels in Structure, Spatial Development, and Timing of Nephrogenesis A2 - Little, Melissa H. In: *Kidney Development, Disease, Repair and Regeneration.* San Diego, Academic Press, 2016, pp 27-40.

4. Osathanondh, V, Potter, E: Development of human kidney as shown by microdissection. III. Formation and interrelationship of collecting tubules and nephrons. *Archives of Pathology,* 76**:** 290-302, 1963.

5. Saxen, L: *Organogenesis of the Kidney,* Cambridge, UK, Cambridge University Press, 1987.

6. Hinchliffe, SA, Sargent, PH, Howard, CV, Chan, YF, Van Velzen, D: Human intrauterine renal growth expressed in absolute number of glomeruli assessed by the disector method and Cavalieri principle. *Lab Invest,* 64**:** 777-784, 1991.

7. Nyengaard, JR, Bendtsen, TF: Glomerular number and size in relation to age, kidney weight, and body surface in normal man. *Anat Rec,* 232**:** 194-201, 1992.

8. Keller, G, Zimmer, G, Mall, G, Ritz, E, Amann, K: Nephron number in patients with primary hypertension. *N Engl J Med,* 348**:** 101-108, 2003.

9. Hoy, WE, Hughson, MD, Zimanyi, M, Samuel, T, Douglas-Denton, R, Holden, L, Mott, S, Bertram, JF: Distribution of volumes of individual glomeruli in kidneys at autopsy: association with age, nephron number, birth weight and body mass index. *Clin Nephrol,* 74 Suppl 1**:** S105-112, 2010.

10. Puelles, VG, Hoy, WE, Hughson, MD, Diouf, B, Douglas-Denton, RN, Bertram, JF: Glomerular number and size variability and risk for kidney disease. *Curr Opin Nephrol Hypertens,* 20**:** 7-15, 2011.

11. Hoy, WE, Hughson, MD, Singh, GR, Douglas-Denton, R, Bertram, JF: Reduced nephron number and glomerulomegaly in Australian Aborigines: a group at high risk for renal disease and hypertension. *Kidney Int,* 70**:** 104-110, 2006.

12. Hughson, MD, Douglas-Denton, R, Bertram, JF, Hoy, WE: Hypertension, glomerular number, and birth weight in African Americans and white subjects in the southeastern United States. *Kidney Int,* 69**:** 671-678, 2006.

13. McNamara, BJ, Diouf, B, Douglas-Denton, RN, Hughson, MD, Hoy, WE, Bertram, JF: A comparison of nephron number, glomerular volume and kidney weight in Senegalese Africans and African Americans. *Nephrol Dial Transplant,* 25**:** 1514-1520, 2010.

14. Cosgrove, M, Goodyer, P: Chapter 9 - Genetic and Epigenetic Regulation of Nephron Number in the Human A2 - Little, Melissa H. In: *Kidney Development, Disease, Repair and Regeneration.* San Diego, Academic Press, 2016, pp 95-102.

15. Hoy, WE, Hughson, MD, Bertram, JF, Douglas-Denton, R, Amann, K: Nephron number, hypertension, renal disease, and renal failure. *J Am Soc Nephrol,* 16**:** 2557-2564, 2005.

16. Ingelfinger, JR: Disparities in renal endowment: causes and consequences. *Adv Chronic Kidney Dis,* 15**:** 107-114, 2008.

17. Takasato, M, Little, MH: The origin of the mammalian kidney: implications for recreating the kidney in vitro. *Development,* 142**:** 1937-1947, 2015.

18. Short, KM, Combes, AN, Lefevre, J, Ju, AL, Georgas, KM, Lamberton, T, Cairncross, O, Rumballe, BA, McMahon, AP, Hamilton, NA, Smyth, IM, Little, MH: Global quantification of tissue dynamics in the developing mouse kidney. *Dev Cell,* 29**:** 188-202, 2014.

19. Osathanondh, V, Potter, EL: Development of Human Kidney as Shown by Microdissection. Ii. Renal Pelvis, Calyces, and Papillae. *Arch Pathol,* 76**:** 277-289, 1963.

20. Oliver, J: Nephrons and Kidneys; A Quantitative Study of Developmental and Evolutionary Mammalian Renal Architectonics. *New York: Hoeber Medical Division Harper & Row*, 1968.

21. Naruse, K, Fujieda, M, Miyazaki, E, Hayashi, Y, Toi, M, Fukui, T, Kuroda, N, Hiroi, M, Kurashige, T, Enzan, H: An immunohistochemical study of developing glomeruli in human fetal kidneys. *Kidney Int,* 57**:** 1836-1846, 2000.

22. Faa, G, Gerosa, C, Fanni, D, Nemolato, S, Locci, A, Cabras, T, Marinelli, V, Puddu, M, Zaffanello, M, Monga, G, Fanos, V: Marked interindividual variability in renal maturation of preterm infants: lessons from autopsy. *J Matern Fetal Neonatal Med,* 23 Suppl 3**:** 129-133, 2010.

23. Sutherland, MR, Gubhaju, L, Moore, L, Kent, AL, Dahlstrom, JE, Horne, RS, Hoy, WE, Bertram, JF, Black, MJ: Accelerated maturation and abnormal morphology in the preterm neonatal kidney. *J Am Soc Nephrol,* 22**:** 1365-1374, 2011.

24. Chikkannaiah, P, Roy, M, Kangle, R, Patil, PV: Glomerulogenesis: can it predict the gestational age? A study of 176 fetuses. *Indian J Pathol Microbiol,* 55**:** 303-307, 2012.

25. Zohdi, V, Sutherland, MR, Lim, K, Gubhaju, L, Zimanyi, MA, Black, MJ: Low Birth Weight due to Intrauterine Growth Restriction and/or Preterm Birth: Effects on Nephron Number and Long-Term Renal Health. *Int J Nephrol,* 2012**:** 136942, 2012.

26. Galinsky, R, Moss, TJ, Gubhaju, L, Hooper, SB, Black, MJ, Polglase, GR: Effect of intra-amniotic lipopolysaccharide on nephron number in preterm fetal sheep. *Am J Physiol Renal Physiol,* 301**:** F280-285, 2011.

27. Hughson, M, Farris, AB, 3rd, Douglas-Denton, R, Hoy, WE, Bertram, JF: Glomerular number and size in autopsy kidneys: the relationship to birth weight. *Kidney Int,* 63**:** 2113-2122, 2003.

28. Verburg, BO, Geelhoed, JJ, Steegers, EA, Hofman, A, Moll, HA, Witteman, JC, Jaddoe, VW: Fetal kidney volume and its association with growth and blood flow in fetal life: The Generation R Study. *Kidney Int,* 72**:** 754-761, 2007.

29. Sampaio, FJB: Analysis of Kidney Volume Growth during the Fetal Period in Humans. *Urological Research,* 20**:** 271-274, 1992.

30. Gloor, JM, Breckle, RJ, Gehrking, WC, Rosenquist, RG, Mulholland, TA, Bergstralh, EJ, Ramin, KD, Ogburn, PL, Jr.: Fetal renal growth evaluated by prenatal ultrasound examination. *Mayo Clin Proc,* 72**:** 124-129, 1997.

31. Konje, JC, Abrams, KR, Bell, SC, Taylor, DJ: Determination of gestational age after the 24th week of gestation from fetal kidney length measurements. *Ultrasound Obstet Gynecol,* 19**:** 592-597, 2002.

32. Chitty, LS, Altman, DG: Charts of fetal size: kidney and renal pelvis measurements. *Prenat Diagn,* 23**:** 891-897, 2003.

33. van Vuuren, SH, Damen-Elias, HA, Stigter, RH, van der Doef, R, Goldschmeding, R, de Jong, TP, Westers, P, Visser, GH, Pistorius, LR: Size and volume charts of fetal kidney, renal pelvis and adrenal gland. *Ultrasound Obstet Gynecol,* 40**:** 659-664, 2012.

34. Gubhaju, L, Sutherland, MR, Yoder, BA, Zulli, A, Bertram, JF, Black, MJ: Is nephrogenesis affected by preterm birth? Studies in a non-human primate model. *Am J Physiol Renal Physiol,* 297**:** F1668-1677, 2009.

35. Kopan, R, Chen, S, Little, M: Nephron progenitor cells: shifting the balance of self-renewal and differentiation. *Curr Top Dev Biol,* 107**:** 293-331, 2014.

36. Rumballe, BA, Georgas, KM, Combes, AN, Ju, AL, Gilbert, T, Little, MH: Nephron formation adopts a novel spatial topology at cessation of nephrogenesis. *Dev Biol,* 360**:** 110-122, 2011.

37. Hartman, HA, Lai, HL, Patterson, LT: Cessation of renal morphogenesis in mice. *Dev Biol,* 310**:** 379-387, 2007.

38. Chen, S, Brunskill, EW, Potter, SS, Dexheimer, PJ, Salomonis, N, Aronow, BJ, Hong, CI, Zhang, T, Kopan, R: Intrinsic Age-Dependent Changes and Cell-Cell Contacts Regulate Nephron Progenitor Lifespan. *Dev Cell,* 35**:** 49-62, 2015.

39. Puelles, VG, Douglas-Denton, RN, Cullen-McEwen, L, McNamara, BJ, Salih, F, Li, J, Hughson, MD, Hoy, WE, Nyengaard, JR, Bertram, JF: Design-based stereological

methods for estimating numbers of glomerular podocytes. *Ann Anat,* 196**:** 48-56, 2014.

40. Rodriguez, MM, Gomez, AH, Abitbol, CL, Chandar, JJ, Duara, S, Zilleruelo, GE: Histomorphometric analysis of postnatal glomerulogenesis in extremely preterm infants. *Pediatr Dev Pathol,* 7**:** 17-25, 2004.

41. Nyengaard, JR, Marcussen, N: The number of glomerular capillaries estimated by an unbiased and efficient stereological method. *J Microsc,* 171**:** 27-37, 1993.

42. Puelles, VG, Cullen-McEwen, LA, Taylor, GE, Li, J, Hughson, MD, Kerr, PG, Hoy, WEAF, Bertram, JF: Human podocyte depletion in association with older age and hypertension. *Am J Physiol Renal Physiol***:** ajprenal 00497 02015, 2016.

43. Puelles, VG, Douglas-Denton, RN, Cullen-McEwen, LA, Li, J, Hughson, MD, Hoy, WE, Kerr, PG, Bertram, JF: Podocyte Number in Children and Adults: Associations with Glomerular Size and Numbers of Other Glomerular Resident Cells. *J Am Soc Nephrol,* 26**:** 2277-2288, 2015.

44. Johnson, JE, Jr., Barrows, CH, Jr.: Effects of age and dietary restriction on the kidney glomeruli of mice: observations by scanning electron microscopy. *Anat Rec,* 196**:** 145-151, 1980.

Figures and Tables

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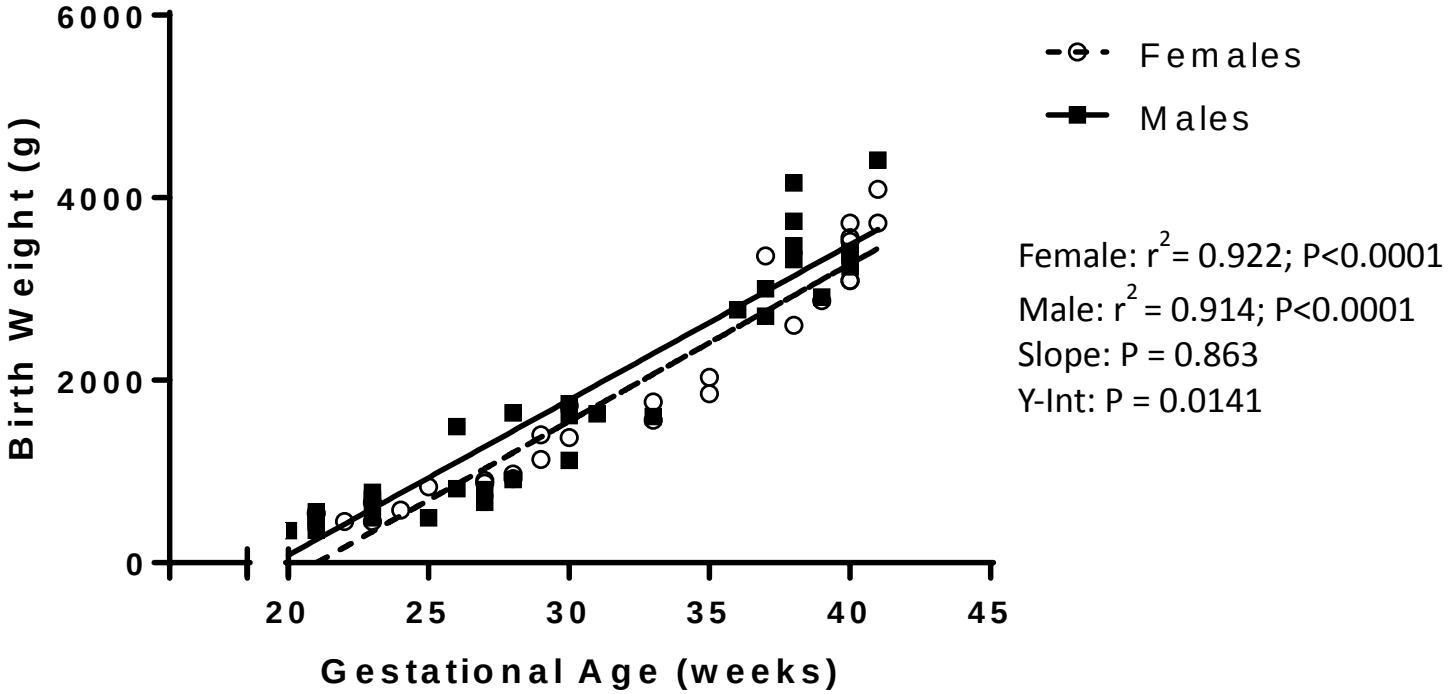

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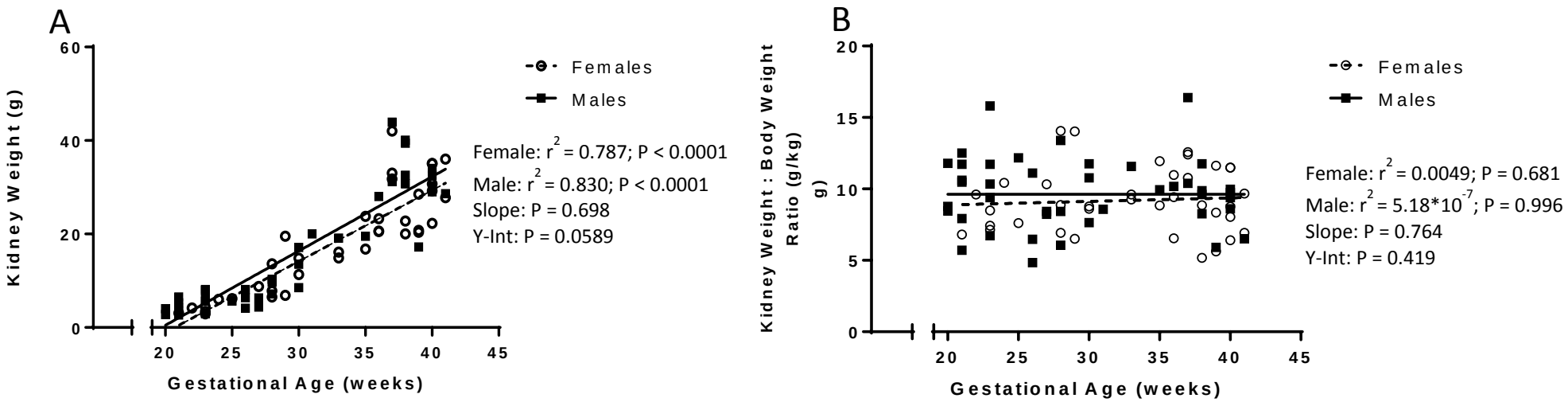

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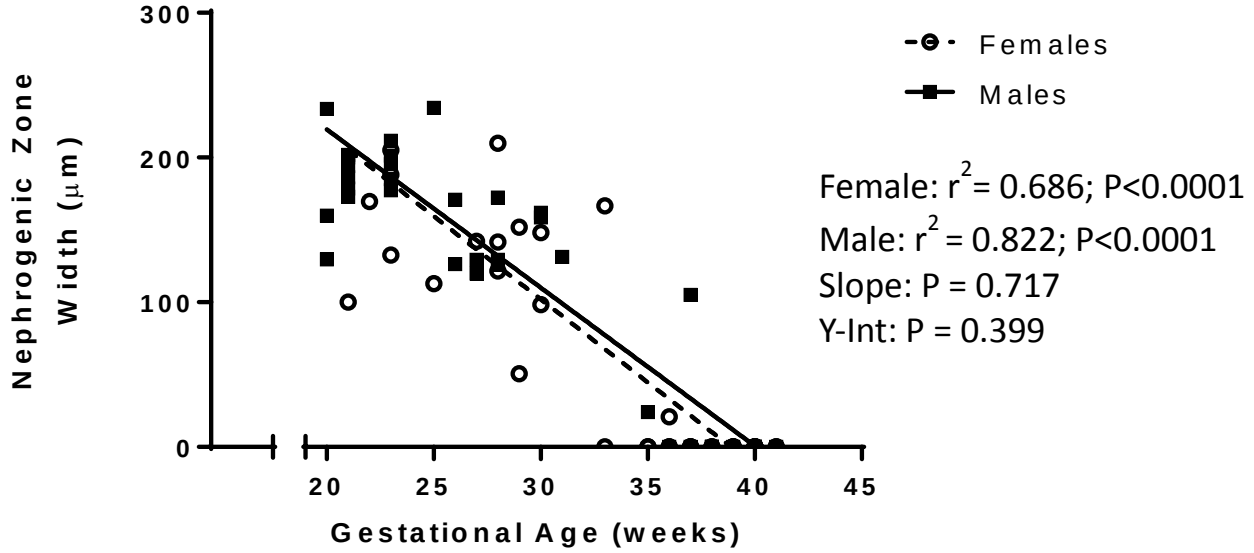

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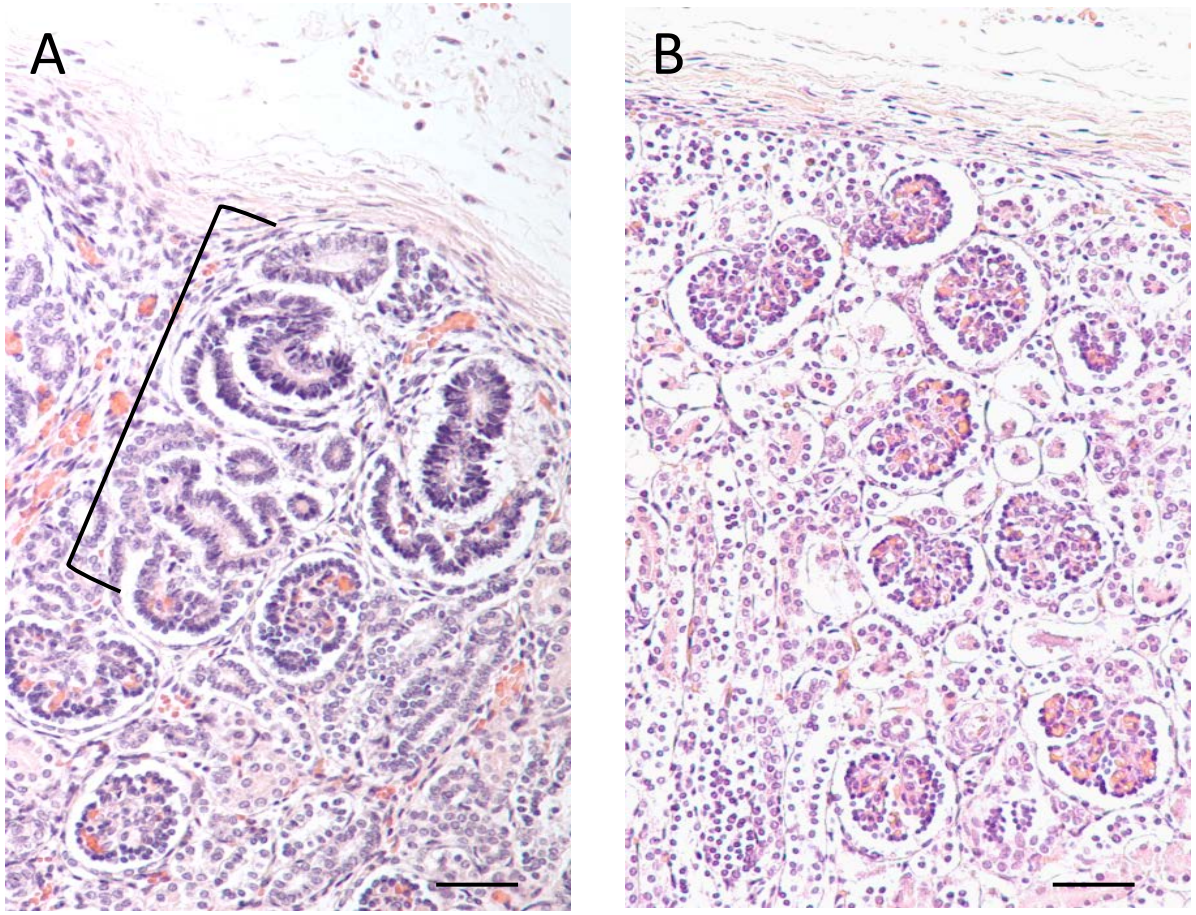

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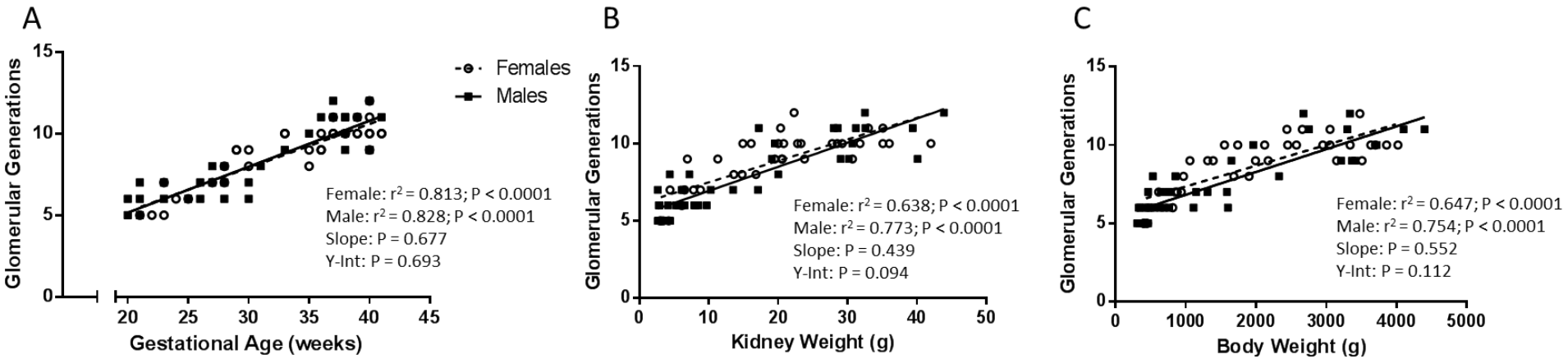

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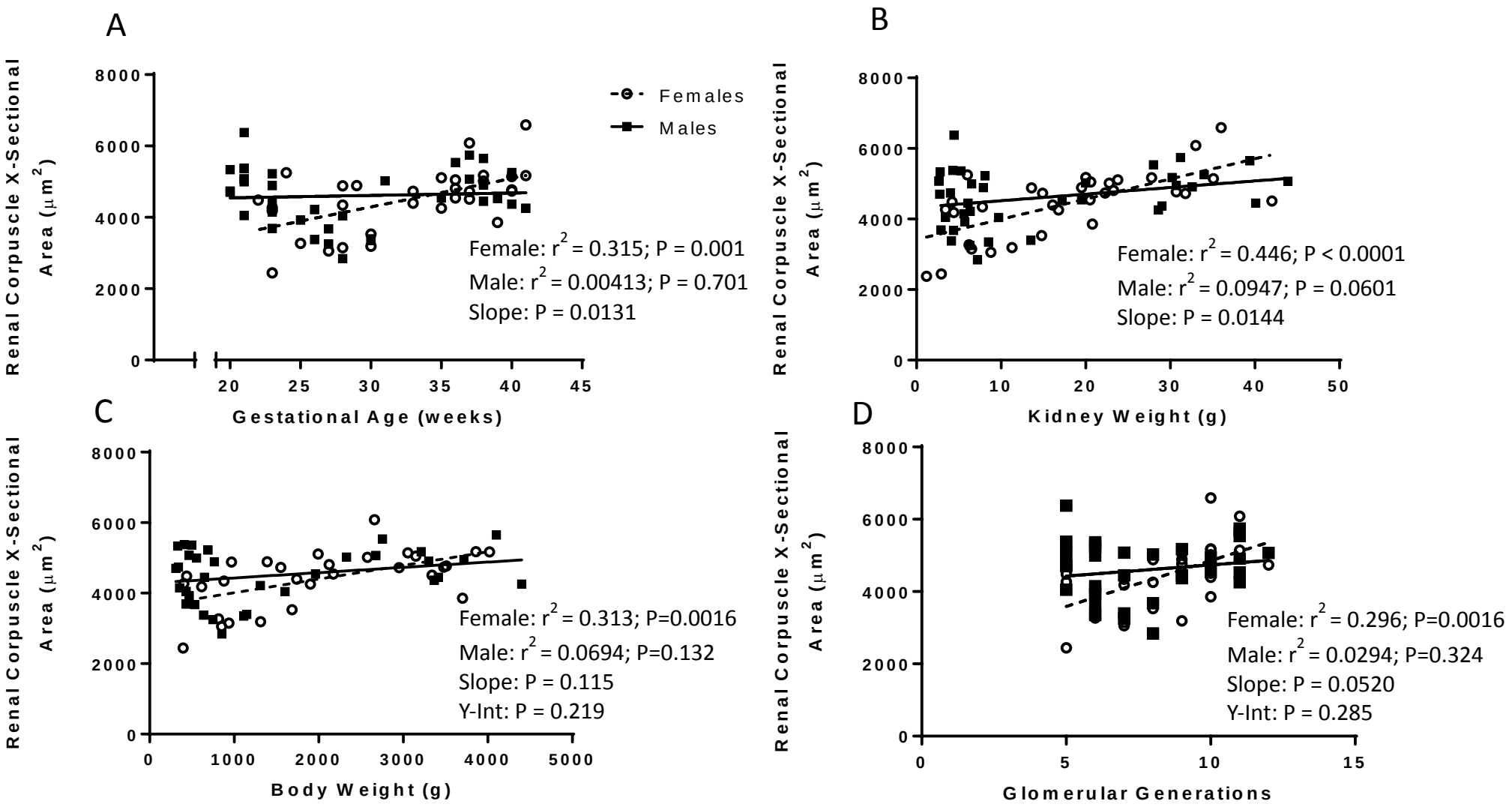

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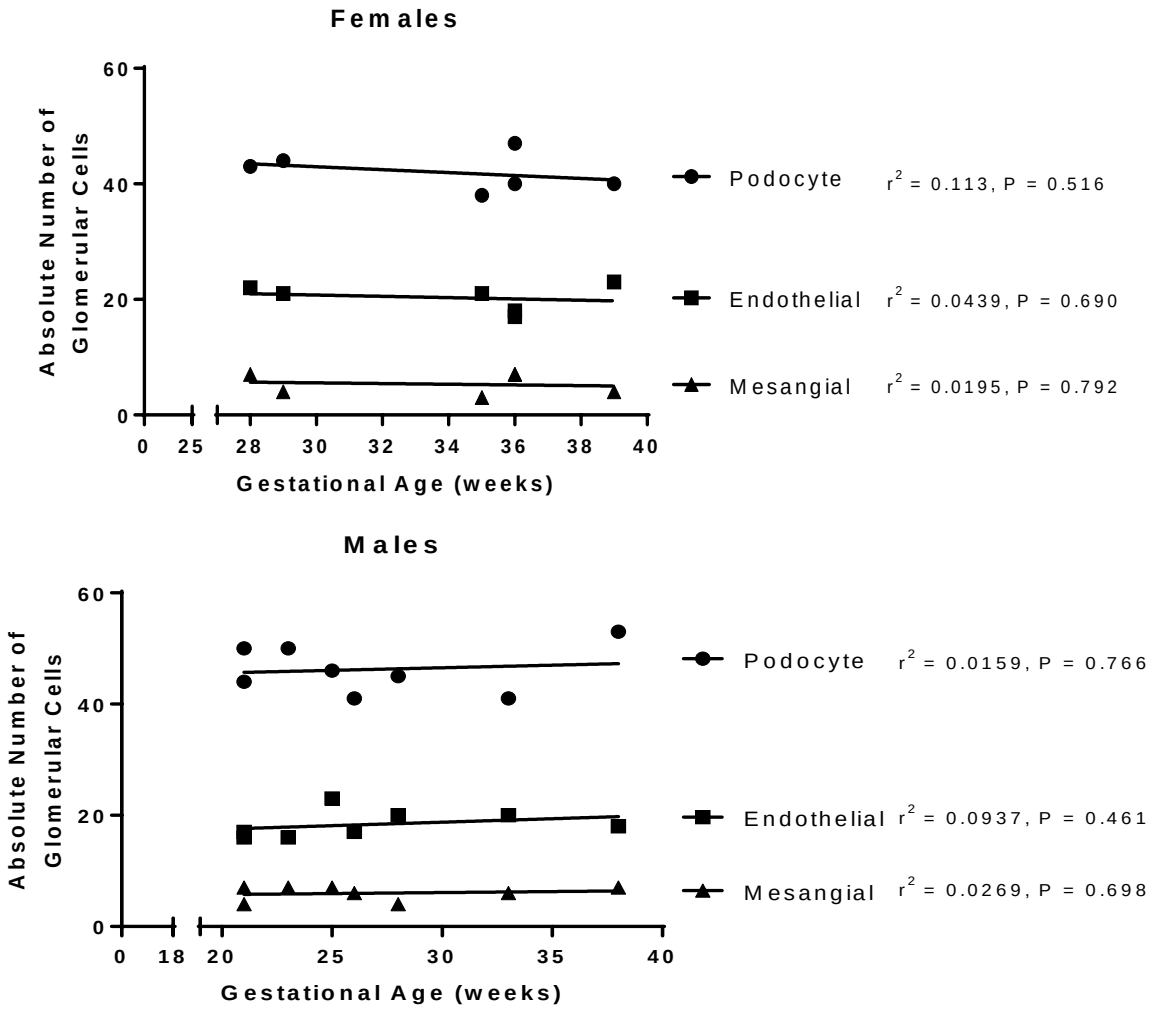

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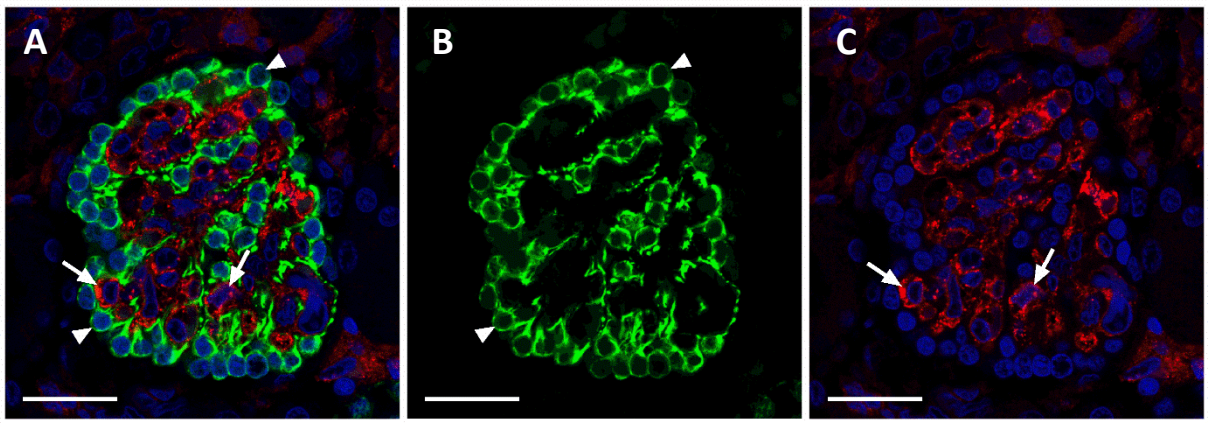

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Gestational Age (weeks)	Sex	Nephrogenesis Ongoing
20-30	F (n=16), M (n=27)	Y
31	M	Y
33	F	N
33	F	Y
33	M	Y
35	F	N
35	F	N
35	M	Y
36	F	N
36	F	Y
36	F	N
36	M	N
37	F	N
37	F	N
37	F	N
37	M	Y
37	M	N
38-41	F (n=12), M (n=10)	N

Chapter Three:
EFFECTS OF PRETERM BIRTH AND VENTILATION ON GLOMERULAR CAPILLARY GROWTH IN THE NOENATAL LAMB KIDNEY

CHAPTER THREE DECLARATION

Declaration by candidate

[It is to be noted that I have used my married surname (Ryan) in this publication]

Chapter 3 was published in the Journal of Hypertension in 2016. Reprinted in this thesis is a copy of the final printed manuscript. Sutherland and Ryan et al. (2016). "Effects of preterm birth and ventilation on glomerular capillary growth in the neonatal lamb kidney." J Hypertens **34** (10) 1988-1997.

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

Nature of contribution	Extent of contribution (%)
I extended the studies of Megan Sutherland (previously written up in her PhD thesis). I have re-analysed the kidneys and I have conducted all of the experimental work (except the animal studies). Additional animals were included and I conducted the analysis of my data. I was co-first author on the manuscript with Megan Sutherland.	50%

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

Name	Nature of contribution	Extent of contribution (%) for student co-authors only	Co-author(s), Monash student Y/N
Megan Sutherland	The initial studies that formed the basis of the experimental work of this chapter were performed by Megan Sutherland and formed a chapter in her PhD thesis. Megan Sutherland is a co-first author on the manuscript.	30%	y
Mar Janna Dahl Kurt Albertine, M. Jane Black	Involved in the study design, obtained funding, performed the animal studies and assisted in editing the manuscript.		N

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

Candidate's Signature	[redacted]	Date 21.9.16
Main Supervisor's Signature		Date 21.9.16

Original Article

Effects of preterm birth and ventilation on glomerular capillary growth in the neonatal lamb kidney

Megan R. Sutherland[a,*], Danica Ryan[a,*], Mar Janna Dahl[b], Kurt H. Albertine[b], and Mary Jane Black[a]

Objectives: Preterm birth is linked to the development of hypertension later in life. This may relate to impaired glomerular capillary growth following preterm birth. The aim of this study was to determine the effects of preterm birth, and/or ventilation, on glomerular capillary growth in the neonatal lamb kidney.

Methods: Four experimental groups were analysed: preterm lambs delivered at 130 days gestation (term = 147 days) and mechanically ventilated for 3 days (preterm ventilated: $n = 9$), 133 days gestational controls (gestational control: $n = 5$), term controls, unassisted breathing for 3 days (term control: $n = 8$), and term lambs ventilated for 3 days (term ventilated: $n = 5$). In perfusion-fixed kidneys, total nephron number, average total capillary length, and surface area per renal corpuscle were stereologically assessed, and total renal filtration surface area (TRFSA) was calculated.

Results: In comparison with term controls, preterm lambs had significantly reduced glomerular capillary length, surface area, and TRFSA, indicative of a low renal functional capacity. Term-ventilated lambs exhibited significantly reduced glomerular capillary length and surface area compared with term controls, indicating that ventilation impairs glomerular capillary growth independently of preterm birth.

Conclusion: Impaired glomerular capillary growth and subsequent reduced TRFSA following preterm birth may mediate the increased predisposition to hypertension later in life.

Keywords: angiogenesis, hypertension, nephrogenesis, preterm birth, renal development, ventilation

Abbreviation: TRFSA, total renal filtration surface area

INTRODUCTION

Over recent decades, there have been many epidemiological studies linking preterm birth (birth prior to 37 weeks gestation) with the development of hypertension later in life [1,2]; increases in SBP of 0.31–0.5 mmHg for each week less than term at birth have been reported [3,4]. The cause of the elevated blood pressure (BP) in subjects born preterm may relate to impaired growth of glomerular capillaries in the neonatal period, and this would adversely impact total renal filtration surface area (TRFSA). A reduced TRFSA is linked to sodium retention and a subsequent elevation in BP [5,6].

When birth comes early, the developmentally immature kidneys are prematurely exposed to the haemodynamic transition that occurs at birth; this includes a marked rise in arterial BP and renal blood flow [7,8]. In addition, the immature kidneys are abruptly exposed to high O_2 concentrations relative to the intrauterine environment through ventilation and supplemental O_2 therapy (21–100% O_2 is utilized [9]) with arterial blood O_2 saturation rapidly increasing after birth [10]. These high O_2 concentrations are known to impact hypoxia-inducible factor (HIF)-1α levels and subsequently downregulate the expression of vascular endothelial growth factor (VEGF) [11,12] which is essential for glomerular capillary growth and development [13,14]. Impaired vascularization in other organ systems following preterm birth is related to O_2 exposure [15].

Mechanical ventilation is often required to facilitate survival after preterm birth; 60–70% of infants born at 22–28 weeks gestation have been reported to be intubated and ventilated after birth [16], and a proportion of infants born moderately preterm or near-term also require mechanical ventilation following respiratory distress [17]. In this study, we hypothesized that preterm birth and mechanical ventilation impair glomerular capillary growth in newborn infants. Utilizing a sheep model of moderate preterm birth, the aim of this study was to examine the effect of moderate preterm birth and/or mechanical ventilation on glomerular capillary length, surface area, and TRFSA.

METHODS

Animals

Studies were conducted using mixed-breed lambs of known delivery dates. Moderately preterm lambs were

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delivered via caesarean section at 130 days gestation (term = 147 days) and ventilated for 3 days after birth (preterm ventilated: $n = 9$). Age-matched gestational (foetal) controls were delivered by caesarean section and euthanized at 133 days gestation (gestational control: $n = 5$). Term lambs were delivered vaginally following spontaneous labour, and they were either nonventilated (term control: $n = 8$) or ventilated for 3 days after birth (term ventilated: $n = 5$). All animals, except the foetal controls, were euthanized at postnatal day 3 (approximately 72 h after birth).

Detailed descriptions of the animal care procedures have been published previously [18,19]. Briefly, preterm lambs were delivered by caesarean section, intubated, and administered 2.5 ml of Survanta (NDC 0074-1040-08; Ross Products Division, Abbott Laboratories, Columbus, Ohio, USA). During resuscitation, preterm lambs received intermittent mandatory ventilation (IMV), with warmed and humidified 100% O_2 (Bird VIP ventilator, model 15215; Bird Products, Palm Springs, California, USA). Respiratory rate was 60 bpm, inspiratory time 0.3 s, positive end-expiratory pressure was set at 8 cmH_2O, and peak inspiratory pressure was adjusted to reach a target $PaCO_2$ of 45–60 mmHg and pH 7.25–7.35. Target expiratory tidal volume was 5–7 ml/kg per breath. At 5–10 min of age, additional Survanta (2.5 ml) was administered, and the concentration of inspired O_2 was then decreased to a target PaO_2 of 60–80 mmHg [18]. The term ventilated groups were delivered naturally and received IMV as described above (except that they were not administered surfactant) [19]. Ventilated lambs were kept prone in a veterinary sling mounted on a radiantly heated bed. Lambs were administered intravenous buprenorphine (0.01 mg/kg; Buprenex, Recritt and Coleman Pharmaceuticals, Richmond, Virginia, USA) and either pentobarbital (3–5 mg/kg; Vet Lab, Lenexa, Kansas, USA) or phenobarbital (10 mg/kg; Wyeth Laboratories, Philadelphia, Pennsylvania, USA) as required for sedation [18,19]. Preterm and term ventilated animals were continuously monitored over the 72 h for temperature, heart rate (HR), BP, FiO_2, and urine output. Arterial blood was sampled hourly to measure $PaCO_2$, PaO_2, blood glucose, and blood pH. At the end of the experimental period, the lambs were euthanized by intravenous infusion of pentobarbital sodium solution (60 mg/kg). 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. Fixed kidneys were cleaned of connective tissue, cut into quarters, and weighed. Two opposing quarters were sliced using a razor blade device into 2-mm thick slices. From these, every fourth slice was selected (beginning from a random starting point). Each slice was then further cut into equal pieces, with 10–12 pieces then sampled using a smooth fractionator approach [20] and embedded in glycolmethacrylate. Glycolmethacrylate blocks were exhaustively sectioned at 20 μm, and every 10th and 11th sections were collected and stained with haematoxylin and eosin [21]. In the remaining 2-mm slices, 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 postfixed in osmium tetroxide before being embedded in epon araldite [22]. Epon araldite blocks were sectioned at 1 μm and stained with toluidine blue. Researchers were blinded to the experimental groups during all kidney analyses.

Stereological estimation of kidney volume, nephron number, renal corpuscle volume, and glomerular tuft volume

The glycolmethacrylate-embedded kidney sections were used in the stereological estimation of kidney volume, nephron number, renal corpuscle volume, and glomerular tuft volume. Every 10th section was viewed using a microfiche reader and an orthogonal grid (2 × 2 cm) superimposed over the projected image. Kidney volume was then estimated using the Cavalieri principle [21,23] with the following equation:

$$V_{\text{kid}}\,(\text{mm}^3) = 2 \times 4 \times 2 \times 10 \times t \times a(p) \times Ps$$

where 2 is the inverse sampling fraction of the whole kidney, 4 is the inverse sampling fraction of kidney slices (1/4 pieces chosen for further sampling), 2 is the inverse sampling fraction of kidney slices chosen to be embedded, 10 is the inverse sampling fraction of kidney sections collected from each block (every 10th section was collected), t is the section thickness (0.02 mm), $a(p)$ is the area associated with each intersecting point on the orthogonal grid, and Ps is the number of intersecting grid points counted for each kidney.

One intact pair of sections from each block was used in the estimation of nephron number, renal corpuscle volume, and glomerular tuft volume using the physical disector/fractionator approach [21,24,25]. Total nephron (glomerular) number in the kidney (N.glom.kid) was determined using the following equations:

$$\text{N.glom.kid} = 2 \times 4 \times 2 \times 10 \times \frac{Ps}{Pf} \times \frac{1}{2fa} \times Q^{-}$$

$$fa = \frac{(P_{\text{kid}} \times a(p)\ \text{physical disector grid})}{(Pf \times a(p)\ \text{volume count grid})}$$

where the numbers represent the inverse tissue sampling fractions (described above), Ps/Pf is the inverse sampling fraction of sections used for the physical disector (Pf is the number of intersecting grid points counted for the sections chosen for analysis, and Ps is the total number of intersecting grid points counted for each kidney), $1/2fa$ is the fraction of the total section area used to count glomeruli, Q^{-} is the total number of glomeruli counted using a physical disector approach, P_{kid} is the total number of points counted per kidney during the physical disector analysis, and $a(p)$ is the area associated with each intersecting point on the orthogonal grids.

Mean renal corpuscle (glomerular tuft plus Bowman's space and capsule) volume (V_{corp}) and mean glomerular tuft volume (V_{glom}) per kidney were estimated using the following equations:

$$V_{corp} \text{ or } V_{glom}(mm^3) = \frac{\text{volume density of the renal corpuscles } (V_v\text{.corp}) \text{ or glomeruli } (V_v\text{.glom})}{\text{numerical density of nephrons in the kidney } (N_{density})}$$

where V_v.corp or V_v.glom

$$= \frac{\text{total number of points overlying the real corpuscles or glomeruli}}{\text{total number of points intersecting the kidney } (P_{kid})}$$

$$N_{density} = \frac{\text{N.glom.kid}}{V_{kid}}$$

In each kidney, the presence or absence of morphologically abnormal glomeruli (exhibiting an enlarged Bowman's space and shrunken glomerular tuft) was also recorded [21].

Estimation of glomerular capillary length, surface area, and total renal filtration surface area

Two glomeruli per epon araldite section (eight glomeruli per inner, mid, and outer cortex; 24 per kidney) were chosen for analysis using an unbiased method [26]. The glomeruli selected were photographed using an 100× oil-immersion lens (Image Pro Plus, v. 6.0; Media Cybernetics, Rockville, Maryland, USA), viewed at a total magnification of 1700×, and imaging software (Adobe Photoshop CS5 Extended, v. 12.0.4; Adobe Systems Inc., San Jose, California, USA) was used to trace the inner margin of the Bowman's capsule and the boundary of the capillary loops within the glomerular tuft. A 15 × 15 mm 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 each glomerulus (Q^-), the number of capillary boundaries that intersect with the horizontal and vertical lines of the superimposed grid (I_{cap}), and the area associated with each grid point $a(p)$ were recorded [22,24].

The following equation was utilized to calculate renal corpuscle cross-sectional area:

$$\text{Renal corpuscle cross-sectional area } (mm^2) = P_{corp} \times a(p)$$

Average capillary length per renal corpuscle was determined using the following equation:

$$\text{Capillary length } (mm) = \text{Lvcap, corp} \times V_{corp}$$

where the capillary length density per corpuscle and mean renal corpuscle volume were calculated by

$$\text{Lvcap, corp } (mm/mm^3) = \frac{(2 \times Q^-)}{(P_{corp} \times a(p))}$$

$$V_{corp}(mm^3) = \frac{V_v\text{.corp.kid}}{N_{density}}$$

where 2 is a constant that accounts for the capillaries being isotropic.

The average capillary surface area per renal corpuscle was determined by the following equation:

$$\text{Capillary surface area } (mm^2) = \text{Svcap, corp} \times V_{corp}$$

where the capillary surface area density per renal corpuscle and mean renal corpuscle volume were calculated by

$$\text{Svcap, corp } (mm^2/mm^3) = \frac{(2 \times I_{cap})}{(P_{corp} \times k \times d)}$$

$$V_{corp}(mm^3) = \frac{V_v\text{.corp.kid}}{N_{density}}$$

where 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.

The TRFSA per kidney was then determined by multiplying the average surface area of capillaries per renal corpuscle by the total number of nephrons in that kidney [22,24].

Glomerular localization of vascular endothelial growth factor

The localization of VEGF in glomeruli was examined using immunohistochemistry (five kidneys per group). Five-micrometre paraffin-embedded sections were dewaxed and antigen retrieval performed using Target Retrieval Solution (Dako, Carpinteria, California, USA). Nonspecific proteins were blocked using a protein blocking agent (Dako). The primary antibody (rabbit monoclonal; Abcam, anti-VEGF, Ab52917, Cambridge, UK) was applied to the sections at a dilution of 1 : 50 and allowed to bind overnight. A biotinylated secondary antibody (goat antirabbit) was subsequently applied (Dako, ready to use Envison+, Glostrup, Denmark) and binding sites identified using diaminobenzidine (Dako). The sections were then counterstained with haematoxylin. The primary antibody was omitted from the negative controls.

The immunolocalization of VEGF was semiquantitated in 50 glomeruli per kidney using a grading system from 0 to 4; glomeruli with no VEGF immunostaining were assigned a score of 0, and glomeruli with high VEGF immunostaining were assigned a score of 4. The renal cortex was systematically sampled so that glomeruli from the inner cortex through to the outer cortex were examined. At the time of analysis, the location of each sampled glomerulus within the renal cortex (inner, middle, and outer) was recorded.

Statistical analysis

Data were analysed using GraphPad Prism (v.5.03 for Windows; GraphPad Software, California, USA), with data presented as the mean ± SEM. Body and kidney weights, nephron number and density, glomerular tuft volume, and TRFSA were compared between groups using a one-way

analysis of variance (ANOVA), followed by a Bonferroni post-hoc test. Glomerular capillary length and surface area were analysed in pairs of groups using a two-way ANOVA, with the factors group (p_G), region of cortex (p_R), and their interaction ($p_{G\times R}$). In the ventilated animals, 12-h averages were taken for each recorded physiological parameter, and data were assessed using a two-way repeated measures ANOVA with the factors group (p_G), time (p_T), and their interaction ($p_{G\times T}$). Linear regression analyses were utilized to examine the relationships between glomerular capillary length, surface area, nephron number, TRFSA, and physiological parameters; this was followed by an analysis of covariance (ANCOVA) to compare the slope of the regression lines between groups. Statistical significance was accepted at P less than 0.05.

RESULTS

Arterial blood gases and cardiovascular function in ventilated animals

FiO_2 was significantly higher in preterm ventilated animals compared with term ventilated, particularly within the first 12 h of life; there was no statistically significant difference between groups in PaO_2 or $PaCO_2$ over the 72 h (Fig. 1a–c). Preterm ventilated lambs had significantly lower SBP (Fig. 1d) and pH (Fig. 1h), but DBP, HR, blood glucose, and urine output were not different from the term ventilated animals (Fig. 1). No animals were hypotensive (SBP < 45 mmHg) at any time point.

Body weight and kidney weight

Body weight was significantly greater in term animals (control and ventilated groups) compared with the preterm ventilated; there was no significant difference between the weight of gestational controls at birth and the preterm ventilated group at postnatal day 3 (Fig. 2a). Absolute kidney weight was significantly greater in the term control and term ventilated groups compared with the preterm and gestational control animals (Fig. 2b), whereas kidney weight relative to body weight (Fig. 2c) was significantly lower in the gestational controls compared with all other preterm and term groups.

Nephron number and kidney volume

During the assessment of nephron number, no glomeruli with overt abnormalities were observed. As shown in Fig. 3a, total nephron number was not significantly different between the four groups ($P = 0.09$). Kidney volume averaged $7954 \pm 927\,mm^3$ in the gestational control, $8559 \pm 716\,mm^3$ in the preterm ventilated, $15\,597 \pm 2568\,mm^3$ in the term ventilated, and $14\,455 \pm 585\,mm^3$ in the term control groups; kidney volume was significantly greater in both the term groups compared with the gestational control and preterm ventilated groups ($P < 0.001$).

Glomerular tuft and renal corpuscle volumes

There was no significant difference in mean glomerular tuft volume between the four groups ($P = 0.96$; Fig. 3b). Average renal corpuscle volume, however, was significantly

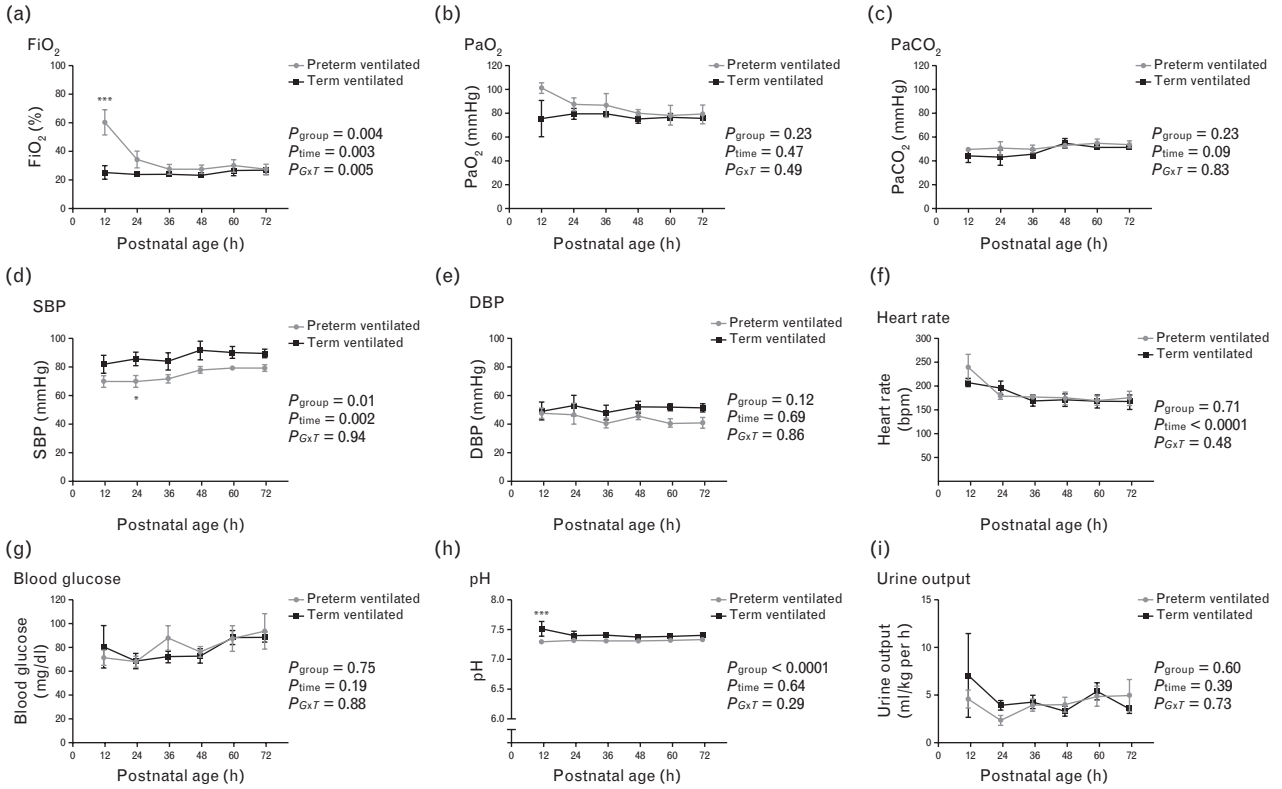

FIGURE 1 Physiological parameters in term and preterm ventilated animals. FiO_2 (a), PaO_2 (b), $PaCO_2$ (c), SBP (d), DBP (e), heart rate (f), blood glucose (g), pH (h), and urine output (i) in preterm ventilated (grey circles) versus term ventilated (black squares) animals over the first 72 h of life. Data assessed by two-way repeated measures analysis of variance with the factors group, time, and their interaction ($p_{G\times T}$). $^*P < 0.05$, $^{***}P < 0.0001$ as determined by Bonferroni post-hoc analysis.

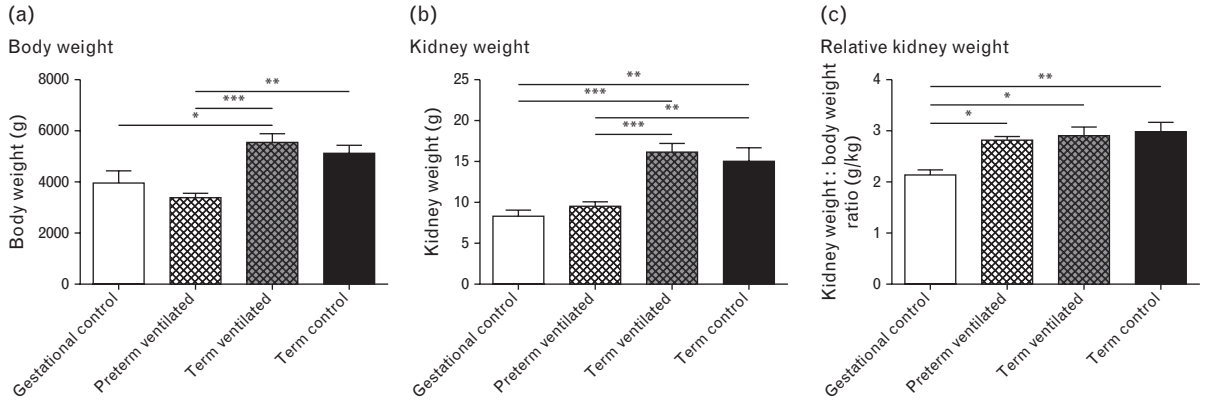

FIGURE 2 Body and kidney weights. Mean body weight (a), kidney weight (b), and kidney to body weight ratio (c) in gestational control animals at 133 days gestation, and preterm ventilated, term ventilated, and term control animals at postnatal day 3. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.0001$ as determined by Bonferroni post-hoc analysis.

larger in the term control animals compared with the preterm ventilated group (Fig. 3c).

Glomerular capillary length and surface area

Representative images of glomeruli from each group are shown in Fig. 4. There was a strong trend for a longer average capillary length per glomerulus in the preterm ventilated lambs compared with the age-matched gestational controls ($P=0.053$; Fig. 5a). Capillary length was significantly reduced in the preterm group compared with the term controls; however, there was no difference between the preterm and term ventilated animals. Term ventilated lambs had markedly reduced average length of capillaries per glomerulus compared with the term controls (Fig. 5a).

Compared with the age-matched gestational controls, preterm lambs had significantly increased average capillary surface area (Fig. 5b). Capillary surface area was significantly lower in the preterm group compared with term controls, but was not different from the term ventilated animals. Term ventilated lambs had a significantly reduced average surface area of capillaries per glomerulus compared with the nonventilated term controls (Fig. 5b).

There was no significant difference between the three regions of cortex with respect to glomerular capillary length or surface area in any of the four groups.

There was a significant positive correlation between average glomerular capillary length and glomerular capillary surface area per kidney in both the preterm ventilated ($r^2=0.63$, $P=0.01$) and term ventilated ($r^2=0.78$, $P=0.048$) groups; however, this relationship was not statistically significant in the gestational control ($r^2=0.55$, $P=0.15$) or term control animals ($r^2=0.32$, $P=0.15$). There were no significant differences in the slope of the regression lines between groups (ANCOVA analysis); therefore, all animals were pooled which revealed a strong positive correlation between average capillary length and surface area (Fig. 5c). Overall, there was no correlation between nephron number and average capillary length or surface area (data not shown). There was also no significant correlation between average glomerular capillary length or surface area and any physiological parameters (data not shown).

Total renal filtration surface area

TRFSA averaged $27\,136 \pm 4504\,mm^2$ in the gestational controls, $32\,249 \pm 2537\,mm^2$ in the preterm ventilated, $37\,403 \pm 7899\,mm^2$ in the term ventilated, and $47\,782 \pm 3515\,mm^2$ in the term control groups. TRFSA in the term control lambs was significantly greater than in both the gestational control and preterm ventilated groups (Fig. 5d). There was no

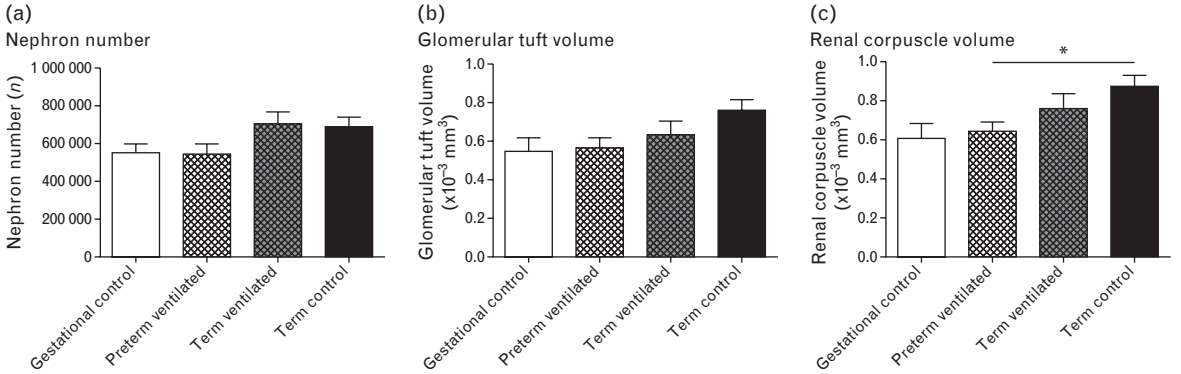

FIGURE 3 Nephron number and renal corpuscle volume. Mean nephron number (a), glomerular tuft volume (b), and renal corpuscle volume (c) in gestational control animals at 133 days gestation, and preterm ventilated, term ventilated, and term control animals at postnatal day 3. $^{*}P<0.05$ as determined by Bonferroni post-hoc analysis.

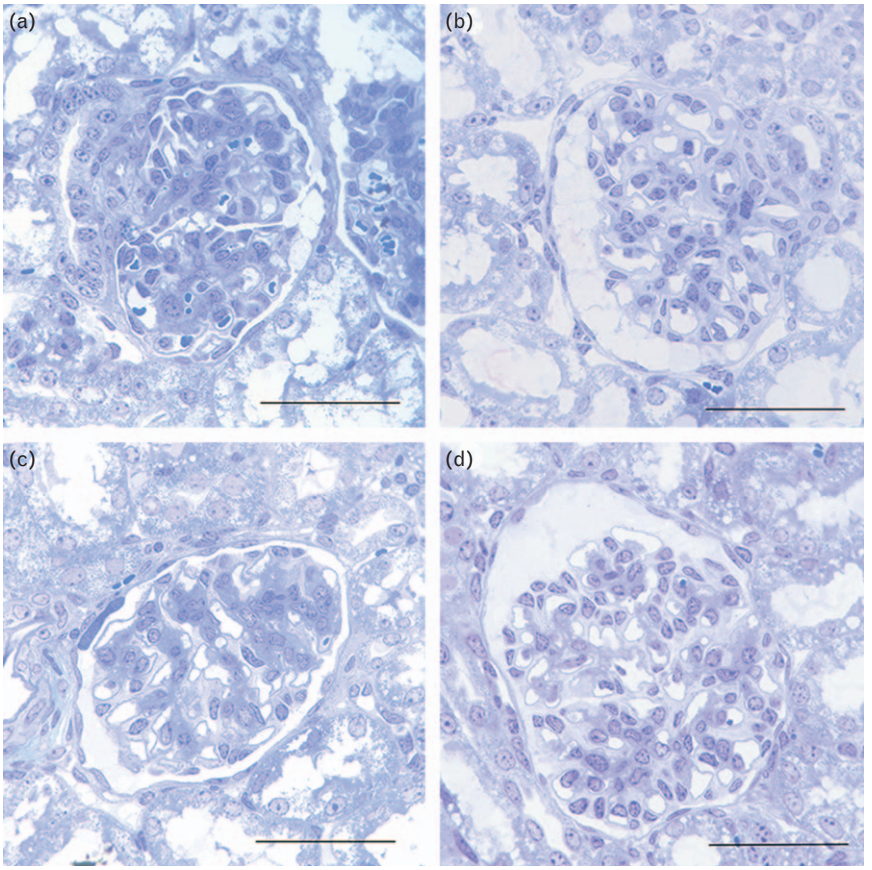

FIGURE 4 Glomerular morphology. Representative photomicrographs of toluidine blue-stained sections of glomeruli from the inner cortex of kidneys from gestational control animals at 133 days gestation (a), preterm ventilated (b), term ventilated (c), and term control (d) animals at postnatal day 3. Scale bar = 50 μm.

significant correlation between TRFSA and any physiological parameters (data not shown).

Glomerular localization of vascular endothelial growth factor

VEGF immunolabelling was clearly observed in the vascular endothelium in all kidney sections. In all kidneys, there was variable VEGF localization in the glomeruli, with high immunolabelling (score of 4) in some glomeruli and no detectable immunolabelling in other glomeruli (score of 0). The variability in VEGF immunolocalization occurred throughout the cortex, with no difference in the proportion of glomeruli with high or low immunolabelling in the inner, middle, and outer regions of the renal cortex. Overall, there was no detectable difference in VEGF immunolabelling in the glomeruli from the four treatment groups with an average grade of 2 in VEGF immunolabelling per glomerulus in the kidneys from the four treatment groups (Fig. 6).

DISCUSSION

Using an ovine model, we have demonstrated that both preterm birth and ventilation are associated with altered glomerular capillary growth. This study carefully modelled the early postnatal clinical treatment of respiratory distress in an infant born moderately preterm; in addition, the impact of mechanical ventilation in a more mature (term) kidney was assessed. Overall, we found that nephron number did not differ between groups; however, TRFSA was significantly reduced in the preterm ventilated animals compared with term controls, indicative of reduced renal functional capacity. Of concern, we have additionally shown that glomerular capillary length and surface area were significantly reduced in the term ventilated animals compared with term controls. These findings suggest that preterm birth (in association with ventilation), as well as ventilation alone, impair glomerular capillary growth in the developing kidney.

Conventional mechanical ventilation is in common use in the clinical treatment of preterm neonates [16,17]. In this study, animals from the preterm and term ventilated groups were ventilated using IMV; all animals were initially exposed to 100% O_2 and then were weaned to lower O_2 concentrations. The reduction in FiO_2 occurred rapidly in the term ventilated animals (with more mature lung function), but the preterm ventilated animals had a longer exposure to significantly higher O_2 concentrations; however, there was no statistically significant difference in PaO_2 between groups. SBP was significantly lower in the preterm lambs compared with those born at term, as is expected because of the strong correlation between BP and both gestational age and body weight [27]. A disadvantage of this study is that the physiological measures were not comparatively assessed in term control animals. Previous studies, however, in term and preterm lambs within the first 3–4 days of life show that all were within the expected range [18,19,28,29].

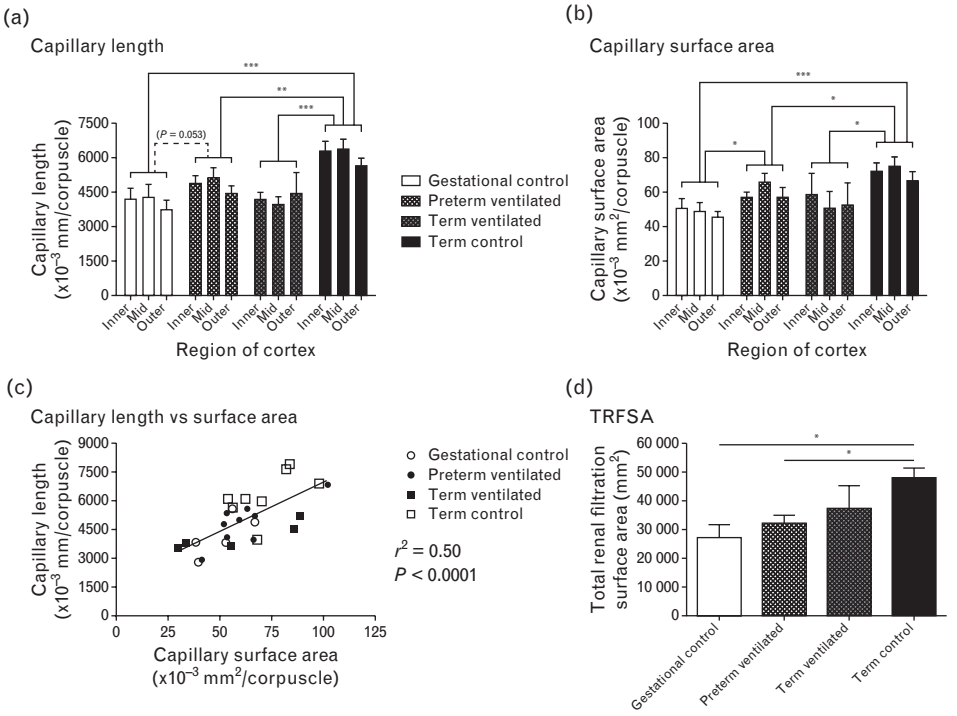

FIGURE 5 Capillary length, surface area, and total renal filtration surface area. Mean capillary length (a) and surface area (b) per renal corpuscle in the inner, mid, and outer regions of the renal cortex in gestational control, preterm ventilated, term ventilated, and term control groups. Data were assessed for every two groups by two-way analysis of variance with the factors group, region of cortex, and their interaction ($p_{G\times R}$). $^{*}P<0.05$, $^{**}P<0.001$, $^{***}P<0.0001$ between groups according to two-way analysis of variance; the factors region of cortex and $p_{G\times R}$ were nonsignificant in all cases. Linear regression analysis of average capillary length versus average capillary surface area per kidney in all animals (c). Total renal filtration surface area (d) in gestational control animals at 133 days gestation, and preterm ventilated, term ventilated, and term control animals at postnatal day 3. $^{*}P<0.05$ as determined by Bonferroni post-hoc analysis.

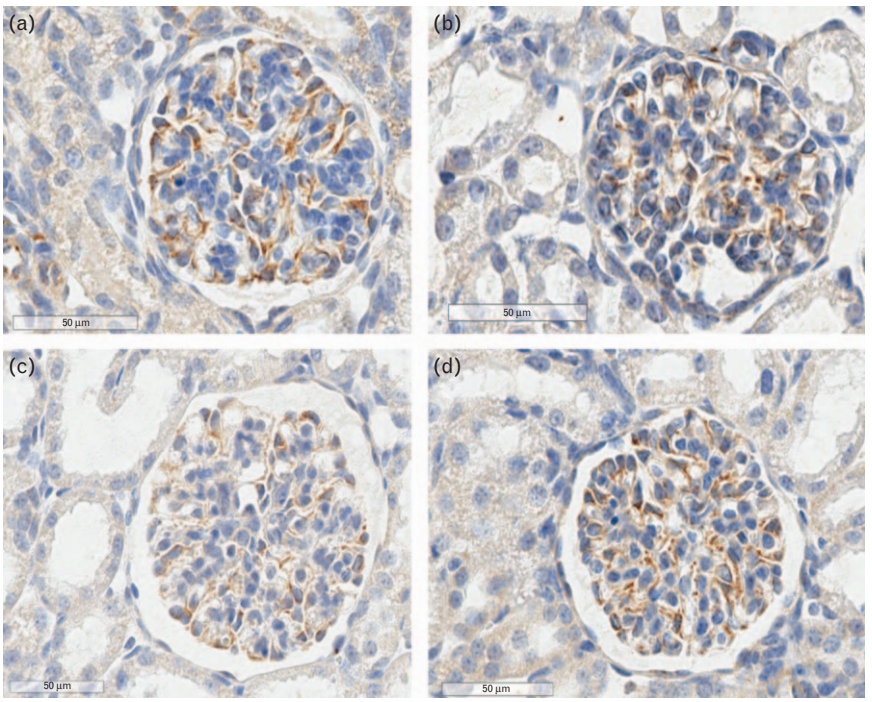

FIGURE 6 Vascular endothelial growth factor immunostaining in glomeruli. Representative vascular endothelial growth factor immunolocalization in glomeruli from gestational control animals at 133 days gestation (a), preterm ventilated (b), term ventilated (c), and term control (d) animals at postnatal day 3. Scale bar = 50 µm.

In a study of kidney development in human neonates [26], we previously demonstrated that preterm birth results in significantly increased renal corpuscle volume (glomerular hypertrophy, possibly indicative of hyperfiltration) compared with age-matched gestational (foetal) controls. In the current study conducted in an ovine model, however, renal corpuscle and tuft volumes were not different between the preterm and gestational control groups. The disparity in findings may relate to the short duration between the time of birth and assessment (3 days) in the current study. Despite there being no overt changes in glomerular size, the preterm lambs did exhibit a significantly increased capillary surface area and a strong trend for increased capillary length compared with the gestational controls. The triggers for capillary growth are unknown, but likely relate to the sudden transition from the intrauterine to the extrauterine environment whereby the immature vasculature needs to quickly adapt to the haemodynamic changes associated with being born (such as significant increases in renal blood flow [7,8]).

We also previously reported the presence of glomeruli exhibiting shrunken glomerular tufts in the kidneys of human and baboon neonates born preterm [26,30–32]. 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 [33]. In the current study, however, no abnormal glomeruli were observed. This finding is not surprising given that nephrogenesis in the sheep is completed by approximately 120 days gestation [34], prior to delivery of the preterm and gestational control animals at 130–133 days gestation in this study; we found no significant difference in nephron number between groups.

Although nephrogenesis was completed, it is possible that glomerular capillary development (glomerulogenesis) was continuing postnatally. In the human foetal kidney, research has shown that the maturation of glomerular endothelial cells is ongoing until approximately 35–39 weeks gestation [35], which follows the completion of nephrogenesis at 32–36 weeks [26,36]. Indeed, the term control lambs exhibited significantly greater capillary length than the gestational controls, which is suggestive of increased growth in relation to increasing age. As the term animals were examined at postnatal day 3, however, it is possible that these differences reflect only postnatal, rather than prenatal, growth. This is supported by the finding of equivalent glomerular capillary length in the preterm ventilated and term ventilated animals at postnatal day 3; it may be speculated that increased capillary growth (as observed in the term controls) may also occur in the preterm kidney in the absence of ventilation. Further studies are certainly required to fully describe the time course of glomerular capillary growth in the developing kidney.

The preterm ventilated lambs had significantly reduced glomerular capillary length, surface area, and renal corpuscle volume compared with term controls (due to increased capillary lengthening and dilatation with increased gestational age in the term animals, and/or to impaired growth of the capillaries after birth in the preterm animals). Importantly, TRFSA was also significantly reduced in the preterm ventilated animals compared with term controls. An important indicator of renal functional capacity, decreased TRFSA implies that renal function was compromised after preterm birth. This is in accordance with the findings of many clinical studies showing that preterm neonates have a much lower glomerular filtration rate compared with neonates born at term [33,37]. In this study, it was not possible to determine whether the lesser glomerular capillary length and surface area in the preterm kidney at postnatal day 3 represents a persistent alteration in glomerular structure. In the long term, however, a low TRFSA would be expected to progressively lead to the development of glomerulosclerosis, nephron loss, hypertension, and ultimately significant renal disease [5,38].

The adverse effects of exposure to high O_2 levels in preterm neonates have been well described in other organ systems [15]; however, to date there have not been any studies that have investigated the effects of ventilation on glomerular vascularization. Studies conducted previously in a rat model of neonatal hyperoxia exposure (80% O_2 P3–P10, a time of ongoing postnatal nephrogenesis) found reduced glomerular size in neonates and a 25% reduction in nephron number in adulthood, suggesting that the kidney is adversely affected [14,39]. Conversely, neonatal hyperoxia exposure (65% O_2) did not have any adverse renal effects in a mouse model [40]. Unlike these rodent models, in this more clinically relevant large animal model, nephrogenesis in the lamb was already completed, mechanical ventilation was used (and for a shorter time period of 3 days), and all animals were initially exposed to 100% O_2 then weaned to lower concentrations.

To determine the effects of ventilation independently of preterm birth, we examined glomerular capillary growth in term-born lambs that had been ventilated for 3 days postnatally. Importantly, a major finding from this study is that early postnatal ventilation appears to impair glomerular capillary growth, with term ventilated animals exhibiting significantly reduced glomerular capillary length and surface area compared with unventilated term controls (similar to the preterm ventilated animals). It should be noted, however, that although capillary length and surface area per renal corpuscle were significantly reduced, this did not translate into a statistically significant difference in TRFSA and thus may not have had an effect on renal functional capacity in the short term. In the long term, however, this impaired vascular development and/or injury may predispose to nephron loss and renal disease [5,38]. As with the preterm ventilated animals, future studies of renal function and morphology in lambs grown to adulthood would be required to determine the long-term impact of neonatal ventilation on renal health.

A possible explanation for the reduced glomerular capillary growth following ventilation is the relative increase in blood O_2 concentrations associated with postnatal ventilation. Indeed, previous studies have shown that in the presence of high-circulating O_2 concentrations, there is prolyl hydroxylase-mediated degradation of the HIF-1α transcription factor [41] and this in turn, leads to down-regulation in the expression of the angiogenic growth factor, VEGF [11,12,42,43]. In the present study, however,

there was no evidence of downregulation of VEGF in the glomeruli of lambs that had been ventilated when compared with those that were not ventilated; thus, the reduced glomerular capillary growth following ventilation does not appear to be mediated via changes in the levels of VEGF expression in the glomeruli. However, there does remain the possibility that the effects on VEGF expression may have occurred acutely when the lambs were initially ventilated and the levels may have normalized by 72 h after birth (the time of necropsy).

Another possible explanation for the impaired glomerular capillary growth following ventilation may relate to changes in renal blood flow (not measured in this study); however, none of the animals were hypotensive suggesting that blood flow was adequate. In addition, given the key role of the glomeruli in blood filtration, there is also the potential for an adverse impact of the sedative and/or analgesic medications on the glomeruli and on glomerular capillary growth. Follow-up studies are required to investigate the mechanisms of normal glomerular capillary growth after birth and why it is impaired following mechanical ventilation.

Given that in this study we have observed negative effects of both preterm birth and postnatal ventilation on the growth of the glomerular capillaries, in future studies it is important to extend these studies to examine the effects of preterm birth on the growth of the vasa recta capillaries. The capillaries of the vasa recta, located in the renal medulla, play a key role in concentrating the urine and importantly, there is experimental evidence indicating that blood flow in the renal medulla is a key factor in maintaining a chronic elevation in BP [44,45]. Hence, impaired growth of the vasa recta capillaries in the preterm neonate may also be a contributing factor to the observed long-term elevation in BP in subjects born preterm.

In conclusion, the findings from this study clearly demonstrate that moderate preterm birth adversely impacts TRFSA and is therefore indicative of reduced renal functional capacity in the preterm neonate. In addition, the findings show that mechanical ventilation, independent of preterm birth, adversely impacts glomerular capillary growth. These adverse effects of preterm birth and/or mechanical ventilation on glomerular capillary growth in the neonate may contribute to the observed elevation in BP in adult subjects that were born preterm. Further studies are required to determine whether the observed impairments in glomerular capillary growth persist into later life.

ACKNOWLEDGEMENTS

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Sutherland MR, Bertagnolli M, Lukaszewski MA, Huyard F, Yzydorczyk C, Luu TM, *et al*. Preterm birth and hypertension risk: the oxidative stress paradigm. *Hypertension* 2014; 63:12–18.
2. de Jong F, Monuteaux MC, van Elburg RM, Gillman MW, Belfort MB. Systematic review and meta-analysis of preterm birth and later systolic blood pressure. *Hypertension* 2012; 59:226–234.
3. Dalziel SR, Parag V, Rodgers A, Harding JE. Cardiovascular risk factors at age 30 following preterm birth. *Int J Epidemiol* 2007; 36:907–915.
4. Johansson S, Iliadou A, Bergvall N, Tuvemo T, Norman M, Cnattingius S. Risk of high blood pressure among young men increases with the degree of immaturity at birth. *Circulation* 2005; 112:3430–3436.
5. Brenner BM, Mackenzie HS. Nephron mass as a risk factor for progression of renal disease. *Kidney Int Suppl* 1997; 63:S124–S127.
6. Brenner BM, Chertow GM. Congenital oligonephropathy and the etiology of adult hypertension and progressive renal injury. *Am J Kidney Dis* 1994; 23:171–175.
7. Paton JB, Fisher DE, Peterson EN, DeLannoy CW, Behrman RE. Cardiac output and organ blood flows in the baboon fetus. *Biol Neonate* 1973; 22:50–57.
8. Aperia A, Broberger O, Herin P, Joelsson I. Renal hemodynamics in the perinatal period. A study in lambs. *Acta Physiol Scand* 1977; 99:261–269.
9. Tan A, Schulze A, O'Donnell CP, Davis PG. Air versus oxygen for resuscitation of infants at birth. *Cochrane Database Syst Rev* 2005; 2:CD002273.
10. Kamlin CO, O'Donnell CP, Davis PG, Morley CJ. Oxygen saturation in healthy infants immediately after birth. *J Pediatr* 2006; 148:585–589.
11. Fujinaga H, Baker CD, Ryan SL, Markham NE, Seedorf GJ, Balasubramaniam V, *et al*. Hyperoxia disrupts vascular endothelial growth factor-nitric oxide signaling and decreases growth of endothelial colony-forming cells from preterm infants. *Am J Physiol Lung Cell Mol Physiol* 2009; 297:L1160–L1169.
12. Maniscalco W, Watkins R, Roper J, Staversky R, O'Reilly M. Hyperoxic ventilated premature baboons have increased p53, oxidant DNA damage and decreased VEGF expression. *Pediatr Res* 2005; 58:549–556.
13. Eremina V, Quaggin SE. The role of VEGF-A in glomerular development and function. *Curr Opin Nephrol Hypertens* 2004; 13:9–15.
14. Popescu CR, Sutherland MR, Cloutier A, Benoit G, Bertagnolli M, Yzydorczyk C, *et al*. Hyperoxia exposure impairs nephrogenesis in the neonatal rat: role of HIF-1alpha. *PLoS One* 2013; 8:e82421.
15. Saugstad OD. Update on oxygen radical disease in neonatology. *Curr Opin Obstet Gynecol* 2001; 13:147–153.
16. Stoll BJ, Hansen NI, Bell EF, Shankaran S, Laptook AR, Walsh MC, *et al*. Neonatal outcomes of extremely preterm infants from the NICHD Neonatal Research Network. *Pediatrics* 2010; 126:443–456.
17. McIntire DD, Leveno KJ. Neonatal mortality and morbidity rates in late preterm births compared with births at term. *Obstet Gynecol* 2008; 111:35–41.
18. Reyburn B, Li M, Metcalfe DB, Kroll NJ, Alvord J, Wint A, *et al*. Nasal ventilation alters mesenchymal cell turnover and improves alveolarization in preterm lambs. *Am J Respir Crit Care Med* 2008; 178:407–418.
19. Bland RD, Xu L, Ertsey R, Rabinovitch M, Albertine KH, Wynn KA, *et al*. Dysregulation of pulmonary elastin synthesis and assembly in preterm lambs with chronic lung disease. *Am J Physiol Lung Cell Mol Physiol* 2007; 292:L1370–L1384.
20. Gundersen HJ. The smooth fractionator. *J Microsc* 2002; 207:191–210.
21. Sutherland MR, Gubhaju L, Black MJ. Stereological assessment of renal development in a baboon model of preterm birth. *Am J Nephrol* 2011; 33 (Suppl 1):25–33.
22. Black M, Briscoe T, Dunstan H, Bertram J, Johnston C. Effect of angiotensin-converting enzyme inhibition on renal filtration surface area in hypertensive rats. *Kidney Int* 2001; 60:1837–1843.
23. Gundersen HJ, Jensen EB. The efficiency of systematic sampling in stereology and its prediction. *J Microsc* 1987; 147 (Pt 3):229–263.
24. Bertram J. Analysing renal glomeruli with the new stereology. *Int Rev Cytol* 1995; 161:111–172.
25. Nyengaard JR. Stereologic methods and their application in kidney research. *J Am Soc Nephrol* 1999; 10:1100–1123.
26. Sutherland MR, Gubhaju L, Moore L, Kent AL, Dahlstrom JE, Horne RS, *et al*. Accelerated maturation and abnormal morphology in the preterm neonatal kidney. *J Am Soc Nephrol* 2011; 22:1365–1374.

27. Lalan S, Blowey D. Comparison between oscillometric and intra-arterial blood pressure measurements in ill preterm and full-term neonates. *J Am Soc Hypertens* 2014; 8:36–44.
28. Beuchee A, Hernandez AI, Duvareille C, Daniel D, Samson N, Pladys P, *et al*. Influence of hypoxia and hypercapnia on sleep state-dependent heart rate variability behavior in newborn lambs. *Sleep* 2012; 35:1541–1549.
29. Pladys P, Arsenault J, Reix P, Rouillard Lafond J, Moreau-Bussiere F, Praud JP. Influence of prematurity on postnatal maturation of heart rate and arterial pressure responses to hypoxia in lambs. *Neonatology* 2008; 93:197–205.
30. Sutherland MR, Gubhaju L, Yoder BA, Stahlman MT, Black MJ. The effects of postnatal retinoic acid administration on nephron endowment in the preterm baboon kidney. *Pediatr Res* 2009; 65:397–402.
31. Gubhaju L, Sutherland MR, Yoder BA, Zulli A, Bertram JF, Black MJ. Is nephrogenesis affected by preterm birth? Studies in a nonhuman primate model. *Am J Physiol Renal Physiol* 2009; 297:F1668–F1677.
32. Sutherland MR, Yoder BA, McCurnin D, Seidner S, Gubhaju L, Clyman RI, *et al*. Effects of ibuprofen treatment on the developing preterm baboon kidney. *Am J Physiol Renal Physiol* 2012; 302:F1286–F1292.
33. Gubhaju L, Sutherland MR, Black MJ. Preterm birth and the kidney: implications for long-term renal health. *Reprod Sci* 2011; 18:322–333.
34. Gimonet V, Bussieres L, Medjebeur AA, Gasser B, Lelongt B, Laborde K. Nephrogenesis and angiotensin II receptor subtypes gene expression in the fetal lamb. *Am J Physiol* 1998; 274 (6 Pt 2):F1062–F1069.
35. Takano K, Kawasaki Y, Imaizumi T, Matsuura H, Nozawa R, Tannji M, *et al*. Development of glomerular endothelial cells, podocytes and mesangial cells in the human fetus and infant. *Toboku J Exp Med* 2007; 212:81–90.
36. Hinchliffe SA, Sargent PH, Howard CV, Chan YF, van Velzen D. Human intrauterine renal growth expressed in absolute number of glomeruli assessed by the disector method and Cavalieri principle. *Lab Invest* 1991; 64:777–784.
37. Gubhaju L, Sutherland MR, Horne RS, Medhurst A, Kent AL, Ramsden A, *et al*. Assessment of renal functional maturation and injury in preterm neonates during the first month of life. *Am J Physiol Renal Physiol* 2014; 307:F149–F158.
38. Hoy WE, Hughson MD, Bertram JF, Douglas-Denton R, Amann K. Nephron number, hypertension, renal disease, and renal failure. *J Am Soc Nephrol* 2005; 16:2557–2564.
39. Yzydorczyk C, Comte B, Cambonie G, Lavoie JC, Germain N, Ting Shun Y, *et al*. Neonatal oxygen exposure in rats leads to cardiovascular and renal alterations in adulthood. *Hypertension* 2008; 52:889–895.
40. Sutherland MR, O'Reilly M, Kenna K, Ong K, Harding R, Sozo F, *et al*. Neonatal hyperoxia: effects on nephrogenesis and long-term glomerular structure. *Am J Physiol Renal Physiol* 2013; 304:F1308–F1316.
41. Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, *et al*. Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. *Science* 2001; 292:468–472.
42. Eremina V, Sood M, Haigh J, Nagy A, Lajoie G, Ferrara N, *et al*. Glomerular-specific alterations of VEGF-A expression lead to distinct congenital and acquired renal diseases. *J Clin Invest* 2003; 111:707–716.
43. Zhang S, Ji Y, Liu X, Lu X, Su W, Zhang D, *et al*. Podocyte-specific VEGF down-regulation and pathophysiological development. *IUBMB Life* 2010; 62:677–683.
44. Cowley AW Jr. Renal medullary oxidative stress, pressure-natriuresis, and hypertension. *Hypertension* 2008; 52:777–786.
45. Cowley AW Jr, Abe M, Mori T, O'Connor PM, Ohsaki Y, Zheleznova NN. Reactive oxygen species as important determinants of medullary flow, sodium excretion, and hypertension. *Am J Physiol Renal Physiol* 2015; 308:F179–F197.

Reviewer's Summary Evaluation

Reviewer 2

This manuscript investigates the consequences of artificial ventilation in preterm lambs on the development of renal glomeruli in the neonatal period. The strength of the investigation is that the experimental challenge has been carefully chosen and rigorously applied with appropriate control studies. This has resulted in novel findings demonstrating how glomerular capillary length, volume, surface area and total renal filtration area were reduced by the ventilation protocol. The weakness is that there is no information as to whether this impairment of glomerular development is transient or whether it persists into later life and may contribute to the genesis of hypertension.

Chapter Four:
A CASE STUDY COMPARING THE CELLULAR COMPOSITION OF GLOMERULI IN INFANTS BORN PRETERM

CHAPTER FOUR DECLARATION

Declaration by candidate
[It is to be noted that I have used my married surname (Ryan) in this manuscript]

Chapter 4 was submitted on the 21st September 2016 in Clinical Science. In this thesis is a copy of the final manuscript.

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

Nature of contribution	Extent of contribution (%)
I conducted all of the experimental work and wrote the manuscript	80%

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

Name	Nature of contribution	Extent of contribution (%) for student co-authors only	Co-author(s), Monash student Y/N
Victor Puelles, Lynette Moore, John Bertram M. Jane Black	Involved in the collection of tissue at autopsy, provided medical records and autopsy reports, involved in the design of experiments, obtained funding and assisted with editing the manuscript		N

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

Candidate's Signature	[redacted]	Date 21.9.16
Main Supervisor's Signature		Date 21.9.16

A CASE STUDY COMPARING THE CELLULAR COMPOSITION OF GLOMERULI IN INFANTS BORN TERM AND PRETERM

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ABSTRACT

Preterm birth (birth before 37 completed weeks of gestation), occurs at a time when the kidneys are structurally and functionally immature. Preterm birth is associated with glomerular hypertrophy in the neonatal period. However, to date little is known as to how glomeruli hypertrophy after preterm birth. Therefore, the aim of this study was to explore the effects of preterm birth on the cellular composition of the glomerulus and in particular glomerular podocyte endowment. Using unbiased stereology and confocal microscopy, the cellular composition of glomeruli from the kidneys of 5 deceased infants (two term stillborn infants and three preterm infants that lived for 5-6 weeks after birth) was examined. The absolute number and relative proportions of the different glomerular cell types (podocytes and non-podocyte cells, comprised of endothelial cells and mesangial cells), as well as the parietal epithelial cells of the Bowman's capsule were measured and compared between groups. Our report shows both renal and glomerular hypertrophy in the two preterm infants, who were of a similar post-conceptional age at the time of autopsy as the stillborn infants born at term. There was little variation in the proportion of podocytes within the glomeruli of all infants, however glomerular hypertrophy in the preterm kidneys was accompanied by a relative decrease in podocyte density within the glomeruli. In this regard, although there appeared to be glomerular hypertrophy in the kidneys of the preterm infants, this did not appear to be the result of cellular proliferation of either podocyte cells or non-podocyte cells; therefore, our findings suggest that glomerular hypertrophy in the preterm kidneys was the result of cellular hypertrophy and/or extracellular matrix deposition.

INTRODUCTION

Preterm birth (defined as birth prior to 37 completed weeks of gestation) occurs at a time when the kidneys are structurally and functionally immature. Preterm delivery can lead to glomerular injury (1, 2) and glomerular hypertrophy (3, 4), with the presence of grossly abnormal glomeruli in the outer renal cortex of some preterm infants (4). Preterm birth occurs at a time when nephrogenesis is still ongoing in the majority of preterm infants, and often occurs during the most active period of nephrogenesis (~60% of nephrons are formed in late gestation) (5). Postnatally, the immature kidneys of the preterm infant need to rapidly adapt to the abrupt haemodynamic changes that occur at birth, including an increase in arterial blood pressure and renal blood flow (6, 7). Furthermore, there is a marked increase in the functional demands of the kidneys as they take over the role of blood filtration postnatally. Likely due to these increased functional demands of the kidneys after birth, preterm birth is subsequently associated with both renal and glomerular hypertrophy (3, 4, 8). The induction of glomerular hypertrophy is of concern, given that it is associated with glomerular hyperfiltration, which can lead to glomerulosclerosis if sustained (9).

To date, little is known as to how glomeruli hypertrophy after preterm birth. The question thus arises, what role does glomerular cell hyperplasia play in this glomerular hypertrophy? A recent study has shown that adult glomerular hypertrophy is associated with increases in the numbers of podocytes and non-podocyte cells, such as endothelial and mesangial cells (10). However, whether or not there is an increase in cell number when glomeruli hypertrophy in the neonatal period after preterm birth is unknown. In this regard, it is imperative to gain an understanding of the effects of preterm birth on the hyperplasia of glomerular podocytes.

Podocytes are highly specialised epithelial cells of the renal glomerulus and their interdigitating foot processes form a crucial component of the glomerular filtration barrier (11-13). Importantly, preterm birth has the potential to adversely impact on podocyte endowment; and if so, this is likely to lead to adverse long-term repercussions to renal health, given that podocyte depletion within glomeruli is a major contributor to the pathogenesis of many renal pathologies (14-17).

There are two proposed mechanisms of podocyte depletion: absolute podocyte depletion and relative podocyte depletion (18). Absolute podocyte depletion occurs when there is loss of podocytes via either apoptosis or detachment from the glomerular basement membrane (reviewed by Tharaux and Huber (19)). Relative podocyte depletion occurs when there is glomerular hypertrophy, such that there are fewer podocytes per unit of glomerular volume (lower podocyte density) (20, 21). Preterm birth has the potential to adversely impact podocyte number via both of these mechanisms. Although nephrogenesis has been shown to continue after preterm birth (if nephrogenesis is ongoing at the time of delivery), it is conceivable that there will be loss of podocytes (as a consequence of the altered haemodynamic and functional demands on the immature kidneys), and/or inadequate postnatal podocyte proliferation during the period of postnatal nephrogenesis.

In this report, we begin to explore the effects of preterm birth on the cellular composition of the glomerulus and in particular glomerular podocyte endowment. Using unbiased stereology , we describe the cellular composition of glomeruli from the kidneys of 5 deceased infants, including the absolute number and relative proportions of the different glomerular cell types (podocytes and non-podocyte cells - NPCs, comprised of endothelial cells and

mesangial cells), as well as the parietal epithelial cells of Bowman's capsule. Of the 5 infants that were studied, 2 were stillborn at term (defined as birth at 37 - 40 weeks' gestational age) and 3 were born prematurely and lived up to 6 weeks after birth.

MATERIALS AND METHODS

Archived neonatal kidneys collected at autopsy were obtained from the Women's and Children's Hospital in North Adelaide, South Australia. Autopsies were performed between the years 1997-2008. Ethics approval for the study of kidneys collected at 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. Written consent for the study of the kidneys for research was obtained by the parents at the time of autopsy.

In this study, kidneys were collected at autopsy from two term stillborn infants born at 39 weeks of gestation and three preterm neonates that were born prematurely (at 27, 32 and 34 weeks of gestation) and lived after birth for 34 days, 42 days and 41 days, respectively. All infants were normally grown *in utero*; they were not exposed to chorioamnionitis and there were no congenital abnormalities at birth. At autopsy, the kidneys of the stillborn infants showed minimal tissue maceration as assessed histologically.

The left kidneys collected at autopsy were weighed and cut into two along the longitudinal plane; large kidneys were further cut transversely. The kidney portions were embedded in paraffin and sectioned at 14µm thickness and up to 20 serial sections were collected. Immunohistochemistry was performed using a "DAKO Cytomation Autostainer Plus" (North America). The protocol developed by Puelles et al. (21) was used to distinguish the different

glomerular cell types using antibody labelling for cell identification (Figure 1). Initially, the kidney sections were rehydrated and subjected to heat-induced antigen retrieval for 30 minutes at 98^{o}C in 10x citrate buffer at pH = 6.0. Podocytes were identified using an antibody against Wilms' Tumour-1 (WT-1) antigen (monoclonal mouse anti-human WT-1-DAKO M356101, clone 6F-H2 [1:50]); the WT-1 antibody has been previously validated in autopsied human kidneys to be cytoplasmic and specific in the staining of podocytes (10, 21, 22). An antibody against von Willebrand Factor (vWF; polyclonal rabbit anti-human vWF-DAKO A008202) [1:200]) was used for the identification of endothelial cells. All nuclei were labelled with 4', 6-diamidino-2-phenylindole (DAPI; 1:10,000 – Sigma–AldrichD9542-10M6).

The immuno-labelled kidney sections were imaged on an Olympus DotSlide system (Olympus, Tokyo, Japan) in order to create a virtual map of the kidney from which 6 mature glomeruli were randomly chosen from the inner renal cortex. The inner cortex was chosen for analysis, since the majority of nephrons in the inner cortex were considered to be morphologically mature in all kidneys (in both the term and preterm infants). The sampled glomeruli were imaged with a Leica SP5 laser confocal microscope (Leica MicroSystems, Mannheim, Germany) and a Z-stack series of 1µm optical sections through each entire sampled glomerulus was obtained using LAS AF Lite software (Leica MicroSystems, Mannheim, Germany). Images were taken using a 40 x objective lens (1.25NA), with a set zoom of (1), using sequential imaging for DAPI (blue cells; 370nm), WT-1 (488nm) and vWF (555nm) at 512 x 512 pixels. The total number of podocytes and non-podocyte cells per glomerulus, and the total number of parietal cells lining Bowman's capsule was then determined using an optical disector stereological method and glomerular volume calculated using the Cavalieri Principle (21).

In addition, a medullary ray glomerular counting method was used to count glomerular generations along 5 clearly defined medullary rays per kidney, and the average number of glomerular generations for each kidney was then determined (3, 4, 23, 24). In two kidneys, nephrogenesis was ongoing at the time of examination (Figure 2), and so in those kidneys, only glomeruli in a relatively mature form were examined; immature glomeruli in the form of comma-shaped and s-shaped bodies were not analysed.

RESULTS

Infant 1. Subject 1 was a normally grown female stillborn infant delivered at 39 weeks' gestational age (term). At delivery, the weight of the infant was 3630 g and crown rump length was 366 mm. The cause of death was attributed to an acute intrauterine asphyxial event, most probably related to uterine rupture. Internal examination revealed normal development of the cardiovascular, respiratory and urogenital systems. Combined kidney weight was 27.80 g (right kidney 13.41 g, left kidney 14.39 g) and the kidneys were normally lobulated. The renal pelvis was not dilated and the ureters and bladder were normal. On histopathological examination of the left kidney there was no evidence of a nephrogenic zone, indicating that nephrogenesis had ceased (Figure 2A). Eleven generations of glomeruli were present and glomerular volume averaged $0.34 \pm 0.06 \times 10^6$ μm^3. The cellular composition of the 6 glomeruli sampled are shown in Table 1, and Figure 3A shows one of the sampled immunolabelled glomeruli. The number of podocytes per glomerulus averaged 296 ± 36 (mean ± SD) cells, comprising 68 ± 5% of the glomerular cells. The average number of non-podocyte cells per glomerulus was 141 ± 42 cells, comprising 32 ± 2% of the glomerular cell types. The average

number of parietal epithelial cells per renal corpuscle was 53 ± 9 cells. Overall, average podocyte density was 871 ± 75 podocytes per 10^6 μm^3.

Infant 2. Subject 2 was a normally grown female stillborn infant delivered at 39 weeks' gestational age (term) with an unexplained cause of death at autopsy. At delivery the weight of the infant was 2970 g and crown rump length was 368 mm. Internal examination revealed normal development of the cardiovascular, respiratory and urogenital systems. Combined kidney weight was 20.88 g (right kidney 10.81 g and left kidney 10.07 g); the ureters and bladder were normally formed. On histopathological examination of the kidneys, nephrogenesis was complete (Figure 2B). There were 10 generations of glomeruli formed within the kidney and glomerular volume averaged 0.27 ± 0.07 x 10^6 μm^3.

The cellular composition of the 6 glomeruli sampled are shown in Table 1, and one of the sampled immunolabelled glomeruli is shown in Figure 3B. The number of podocytes per glomerulus averaged 355 ± 82 cells, comprising 62 ± 2% of the glomerular cells. The average number of total non-podocyte cells was 219 ± 53 cells, comprising 38 ± 5% of glomerular cells. The average number of parietal epithelial cells per renal corpuscle was 71 ± 24 cells. Overall the average podocyte density was 1375 ± 378 cells per 10^6 μm^3.

Infant 3. Subject 3 was a normally grown female infant born at 34 weeks' gestational age. The infant lived for 41 days after birth and died at 40 weeks' gestational equivalent age (term-equivalent age). At birth, the infant weighed 2447 g and at autopsy, the infant weighed 3422 g with a crown rump length of 404 mm. The cause of death was consistent with congestive cardiac failure.

At the time of autopsy, the genitourinary system appeared normal, with normal renal lobulation and a combined kidney weight of 50 g; individual weights of the right and left kidneys were not recorded. The renal cortex and medulla were well demarcated and appeared normal. No focal lesions were apparent. The renal papillae were intact and there was no evidence of parenchymal scarring. The renal pelvises and ureters did not appear dilated and drained to an unremarkable bladder, which was normally situated.

Histopathological examination of the kidneys revealed a normal cortex and medulla with good corticomedullary definition. The kidneys showed cessation of nephrogenesis at the time of autopsy (Figure 2C) and 11 generations of glomeruli were present. Glomerular volume averaged $0.41 \pm 0.07 \times 10^6$ μm^3.

The cellular composition of the 6 glomeruli sampled are shown in Table 1, and Figure 3C shows one of the sampled immunolabelled glomeruli. The number of podocytes per glomerulus averaged 338 ± 47 cells, comprising 59 ± 4% of glomerular cells. The average number of total non-podocyte cells was 241 ± 56 cells, compromising 41 ± 4% of glomerular cells. The average number of parietal cells was 58 ± 13 cells per renal corpuscle. Overall, the average podocyte density was 834 ± 77cells per 10^6 μm^3.

Infant 4: Subject 4 was a normally grown male infant born at 32 weeks' gestational age. The infant lived for 42 days after birth and died at 38 weeks' gestational equivalent age (term equivalent age). At autopsy the infant weighed 2416 g and crown rump length was 465 mm. Birth weight was not recorded in the autopsy report. The cause of death was attributed to respiratory complications following asphyxia at birth. At autopsy, the combined weight of the left and right kidneys was 41 g; individual weights of the right and left kidney were not

recorded. There was some evidence of renal congestion; however, overall, the kidneys showed normal fetal lobulation and no obvious abnormalities. There was normal architectures of the cortex and medulla with no casts or inflammatory cells present.

Histopathological examination of the kidneys showed that nephrogenesis was nearing completion, but not fully complete, with a thin nephrogenic zone still visible in the outer cortex (Figure 2D). Eleven generations of glomeruli were present and average glomerular volume was 0.48 ± 0.05 x 10^6 μm^3. The cellular composition of the 6 glomeruli sampled are shown in Table 1 and Figure 3D shows one of the sampled immunolabelled glomeruli. The number of podocytes per glomerulus averaged 379 ± 18 cells, comprising 63 ± 1% of glomerular cells. The average number of non-podocyte cells was 227 ± 34 cells, comprising 37 ± 3% of glomerular cells. The average number of parietal epithelial cells was 73 ± 14 cells per renal corpuscle. Average podocyte density was 798 ± 85 per 10^6 μm^3.

Infant 5: Subject 5 was a small for gestational age twin male infant, born at 27 weeks' gestational age. The infant lived for 34 days after birth and died at 32 weeks' gestational equivalent age. At delivery the infant weighed 470 g and increased weight at autopsy to 928 g. The crown rump length at the time of death was 230 mm. The cause of death was attributed to complications associated with prematurity, including shock due to necrotizing enterocolitis. Internal examination revealed normal development of the cardiovascular, respiratory and urogenital systems. Combined kidney weight was 8.3 g (right kidney: 4.0 g; left kidney: 4.3 g). There was evidence of normal fetal lobulation, and the cortex and medulla were well demarcated with no apparent focal lesions. The renal pelvis and ureter were not dilated and drained to an unremarkable bladder, which was normally situated.

The cellular composition of the 6 glomeruli sampled are shown in Table 1, and one of the sampled immunolabelled glomeruli is shown in Figure 3E. Histopathological examination of the kidneys showed ongoing nephrogenesis (Figure 2E), with 9 generations of glomeruli present. Average glomerular volume was 0.31 ± 0.08 x 10^6 μm^3. The number of podocytes per glomerulus averaged 267 ± 58 cells, comprising 61 ± 3% of glomerular cells. The average number of non-podocyte cells was 167 ± 35 cells, comprising 39 ± 3% of glomerular cells. The average number of parietal epithelial cells was 69 ± 16 cells per renal corpuscle. Average podocyte density was 877 ± 169 cells per 10^6 μm^3.

DISCUSSION

This is the first paediatric case study, to our knowledge, to have compared the cellular composition and podocyte endowment in glomeruli of preterm and term human infants using design-based stereology (21). Our case reports show both renal and glomerular hypertrophy in the two preterm infants who were of a similar post-conceptional age at the time of autopsy as the stillborn infants born at term; this was evidenced by increased kidney weights and glomerular volume measurements, respectively. The findings, although preliminary, do not support the concept that the glomerular hypertrophy is due to an increase in the number of podocytes or in the number of non-podocyte cells within the glomerulus.

There was considerable variability in the absolute number of podocytes in the sampled glomeruli (all taken from the inner cortical region). This was particularly evident in some infants; ranging from 215 to 459 podocytes per glomerulus in one of the term infants (Infant 2) and 214 to 338 podocytes per glomerulus in one of the preterm infants (Infant 5). Interestingly, however, the relative proportion of podocytes within the glomeruli of all infants

was much less variable. In the two term born infants, podocytes accounted for 68% of glomerular cells in Infant 1, and 62% in Infant 2. In the preterm kidneys, the relative proportion of podocytes per glomerulus was 59% and 63% in the two preterm infants that were term equivalent age at the time of autopsy (Infants 3 and 4, respectively), and 61% in the infant that died at 32 weeks' gestational equivalent age (Infant 5).

Using similar design-based stereology, in previous analyses of four term born children (≤ 3 years old) there was on average 452 podocytes, 389 non-podocyte cells and 146 parietal epithelial cells per glomerulus (10). In the present study, average podocyte number for the four infants whose post-conceptional age at autopsy was between 38 and 40 weeks (Infants 1 to 4) was 342 $\pm$ 35. Compared with the podocyte counts of Puelles et al. (10), this suggests that podocyte number increases by around 34% during early childhood. The source of the additional podocytes is unknown, but given that podocytes are believed to exit the cell cycle following birth (25), it has been proposed that they may originate from the parietal epithelial cells (26, 27). Indeed, there are a number of studies suggesting that an increase in the number of podocytes in glomeruli in postnatal life is mediated by migration of parietal epithelial cells into the glomeruli and their trans-differentiation into podocytes (28). Interestingly, comparison of the present counts of parietal epithelial cells in Infants 1 to 4, (64 + 17 per renal corpuscle) with those of Puelles et al. (10) (146 per renal corpuscle) reveals a marked increase in the number of parietal epithelial cells in early childhood. It is therefore tempting to speculate that the marked increase in parietal cells postnatally may act as a cellular reserve, whereby there is potential for the parietal cells to transdifferentiate into podocytes if required. Likewise, comparison of the glomerular counts of Puelles et al. (10), also indicate a

significant increase in the non-podocyte cellular compartment (endothelial and mesangial cells) in early childhood. In this regard, in previous studies in sheep, we have shown marked postnatal growth of the glomerular capillaries as a result of being born (29), which supports the concept of endothelial proliferation of the glomerular capillaries as the functional demands on the kidney are increased.

Overall, glomerular hypertrophy in preterm kidneys was accompanied by a relative decrease in podocyte density within the glomeruli. This reduction in podocyte density may be of concern, given that a reduced podocyte density is directly linked to vulnerability to renal disease processes (30-32); in particular, glomerulosclerosis (17, 31-37). Hence, the kidneys of preterm infants may be more prone to the development of glomerulosclerosis in later life. Indeed, studies in adolescents and adults have previously demonstrated a link between low birthweight / preterm birth and the later development of focal segmental glomerulosclerosis (FSGS) (38, 39). Furthermore, Ikezumi et al. (39) reported a significant reduction in both absolute podocyte number and density in the glomeruli of FSGS patients born of low birth weight and preterm. Such findings certainly warrant further investigation.

In the preterm infants that died at term-equivalent age (Infants 3 and 4), 11 generations of glomeruli were present, with nephrogenesis not fully complete in one of these infants. This was similar to findings in the two term stillborn infants (where nephrogenesis was completed), with 10 (Infant 2) and 11 (Infant 1) glomerular generations present. In the kidneys of the preterm infant that was born at 27 weeks' gestation and died at 32 weeks' post-conceptional age (Infant 5), 9 glomerular generations were present and nephrogenesis was ongoing. Collectively, our findings are in accordance with previous studies in preterm infants (3, 4) and

non-human primates (40), that have shown that nephrogenesis continues after preterm birth, when nephrogenesis was ongoing at the time of delivery. Our findings also suggest that preterm birth does not adversely impact the number of glomerular generations formed, and this supports our previous findings (4). In contrast, Faa et al. (24) reported a reduction in the number of glomerular generations in deceased preterm neonates relative to term infants; however, many of the preterm infants in that study were intrauterine growth restricted, which is likely to have adversely impacted nephrogenesis.

CONCLUSION

In conclusion, the findings of the present study suggest that podocyte number increases in the early postnatal period, as do numbers of non-podocyte cells and parietal epithelial cells. However, given the small number of cases studied and the small number of glomeruli sampled, it is not possible to make definitive conclusions about glomerular cell numbers when comparing preterm and term infants. Overall however, the findings suggest that the glomerular hypertrophy observed in the kidneys of preterm infants is not the result of cellular proliferation and therefore, it is likely attributed to cellular hypertrophy and/or extracellular matrix deposition.

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REFERENCES

1. Gubhaju L, Sutherland MR, Horne RS, Medhurst A, Kent AL, Ramsden A, et al. Assessment of renal functional maturation and injury in preterm neonates during the first month of life. American journal of physiology Renal physiology. 2014;307(2):F149-58.

2. Tsukahara H, Hiraoka M, Mayumi M. Many confounding factors influencing urinary beta(2)-microglobulin excretion in premature newborns. J Pediatr-Us. 2004;144(6):835-6.

3. Rodriguez MM, Gomez AH, Abitbol CL, Chandar JJ, Duara S, Zilleruelo GE. Histomorphometric analysis of postnatal glomerulogenesis in extremely preterm infants. Pediatric and developmental pathology : the official journal of the Society for Pediatric Pathology and the Paediatric Pathology Society. 2004;7(1):17-25.

4. Sutherland MR, Gubhaju L, Moore L, Kent AL, Dahlstrom JE, Horne RS, et al. Accelerated maturation and abnormal morphology in the preterm neonatal kidney. Journal of the American Society of Nephrology : JASN. 2011;22(7):1365-74.

5. Hinchliffe SA, Lynch MR, Sargent PH, Howard CV, Van Velzen D. The effect of intrauterine growth retardation on the development of renal nephrons. British journal of obstetrics and gynaecology. 1992;99:296-301.

6. Fawer CL, Torrado A, Guignard JP. Maturation of renal function in full-term and premature neonates. Helv Paediatr Acta. 1979;34(1):11-21.

7. Arant BS, Jr. Postnatal development of renal function during the first year of life. Pediatr Nephrol. 1987;1(3):308-13.

8. Huang HP, Tsai IJ, Lai YC, Cheng CH, Tsau YK. Early postnatal renal growth in premature infants. Nephrology. 2007;12(6):572-5.

9. D'Agati V. Pathologic classification of focal segmental glomerulosclerosis. Seminars in nephrology. 2003;23(2):117-34.

10. Puelles VG, Douglas-Denton RN, Cullen-McEwen LA, Li J, Hughson MD, Hoy WE, et al. Podocyte Number in Children and Adults: Associations with Glomerular Size and Numbers of Other Glomerular Resident Cells. Journal of the American Society of Nephrology : JASN. 2015;26(9):2277-88.

11. Rodewald R, Karnovsky MJ. Porous substructure of the glomerular slit diaphragm in the rat and mouse. The Journal of cell biology. 1974;60(2):423-33.

12. Patrakka J, Tryggvason K. Molecular make-up of the glomerular filtration barrier. Biochemical and biophysical research communications. 2010;396(1):164-9.

13. Scott RP, Quaggin SE. Review series: The cell biology of renal filtration. The Journal of cell biology. 2015;209(2):199-210.

14. Meyer TW, Bennett PH, Nelson RG. Podocyte number predicts long-term urinary albumin excretion in Pima Indians with Type II diabetes and microalbuminuria. Diabetologia. 1999;42(11):1341-4.

15. Matsusaka T, Xin J, Niwa S, Kobayashi K, Akatsuka A, Hashizume H, et al. Genetic engineering of glomerular sclerosis in the mouse via control of onset and severity of podocyte-specific injury. Journal of the American Society of Nephrology : JASN. 2005;16(4):1013-23.

16. Wharram BL, Goyal M, Wiggins JE, Sanden SK, Hussain S, Filipiak WE, et al. Podocyte depletion causes glomerulosclerosis: diphtheria toxin-induced podocyte depletion in rats expressing human diphtheria toxin receptor transgene. Journal of the American Society of Nephrology : JASN. 2005;16(10):2941-52.

17. Pagtalunan ME, Miller PL, Jumping-Eagle S, Nelson RG, Myers BD, Rennke HG, et al. Podocyte loss and progressive glomerular injury in type II diabetes. The Journal of clinical investigation. 1997;99(2):342-8.

18. Puelles VG, Bertram JF. Counting glomeruli and podocytes: rationale and methodologies. Current opinion in nephrology and hypertension. 2015;24(3):224-30.

19. Tharaux PL, Huber TB. How many ways can a podocyte die? Seminars in nephrology. 2012;32(4):394-404.

20. Lemley KV, Bertram JF, Nicholas SB, White K. Estimation of Glomerular Podocyte Number: A Selection of Valid Methods. Journal of the American Society of Nephrology. 2013;24(8):1193-202.

21. Puelles VG, Douglas-Denton RN, Cullen-McEwen L, McNamara BJ, Salih F, Li J, et al. Design-based stereological methods for estimating numbers of glomerular podocytes. Annals

of anatomy = Anatomischer Anzeiger : official organ of the Anatomische Gesellschaft. 2014;196(1):48-56.

22. Puelles VG, Cullen-McEwen LA, Taylor GE, Li J, Hughson MD, Kerr PG, et al. Human podocyte depletion in association with older age and hypertension. American journal of physiology Renal physiology. 2016:ajprenal 00497 2015.

23. Hinchliffe SA, Sargent PH, Howard CV, Chan YF, Van Velzen D. Human intrauterine renal growth expressed in absolute number of glomeruli assessed by the disector method and Cavalieri principle. Laboratory investigation; a journal of technical methods and pathology. 1991;64(6):777-84.

24. Faa G, Gerosa C, Fanni D, Nemolato S, Locci A, Cabras T, et al. Marked interindividual variability in renal maturation of preterm infants: lessons from autopsy. The journal of maternal-fetal & neonatal medicine : the official journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstet. 2010;23 Suppl 3:129-33.

25. Wanner N, Hartleben B, Herbach N, Goedel M, Stickel N, Zeiser R, et al. Unraveling the role of podocyte turnover in glomerular aging and injury. Journal of the American Society of Nephrology : JASN. 2014;25(4):707-16.

26. Sagrinati C, Netti GS, Mazzinghi B, Lazzeri E, Liotta F, Frosali F, et al. Isolation and characterization of multipotent progenitor cells from the Bowman's capsule of adult human kidneys. Journal of the American Society of Nephrology : JASN. 2006;17(9):2443-56.

27. Appel D, Kershaw DB, Smeets B, Yuan G, Fuss A, Frye B, et al. Recruitment of podocytes from glomerular parietal epithelial cells. Journal of the American Society of Nephrology. 2009;20(2):333-43.

28. Puelles VG, Moeller MJ. Chapter 24 - Postnatal Cell Turnover in the Nephron Epithelium: What Can This Tell Us? A2 - Little, Melissa H. Kidney Development, Disease, Repair and Regeneration. San Diego: Academic Press; 2016. p. 319-33.

29. Sutherland MR, Ryan D, Dahl MJ, Albertine KH, Black MJ. Effects of preterm birth and ventilation on glomerular capillary growth in the neonatal lamb kidney. Journal of hypertension. 2016.

30. Dalla Vestra M, Masiero A, Roiter AM, Saller A, Crepaldi G, Fioretto P. Is podocyte injury relevant in diabetic nephropathy? Studies in patients with type 2 diabetes. Diabetes. 2003;52(4):1031-5.

31. Wiggins RC. The spectrum of podocytopathies: a unifying view of glomerular diseases. Kidney international. 2007;71(12):1205-14.

32. Fukuda A, Chowdhury MA, Venkatareddy MP, Wang SQ, Nishizono R, Suzuki T, et al. Growth-dependent podocyte failure causes glomerulosclerosis. Journal of the American Society of Nephrology : JASN. 2012;23(8):1351-63.

33. Kriz W, Gretz N, Lemley KV. Progression of glomerular diseases: is the podocyte the culprit? Kidney international. 1998;54(3):687-97.

34. Kim YH, Goyal M, Kurnit D, Wharram B, Wiggins J, Holzman L, et al. Podocyte depletion and glomerulosclerosis have a direct relationship in the PAN-treated rat. Kidney international. 2001;60(3):957-68.

35. Lemley KV, Lafayette RA, Safai M, Derby G, Blouch K, Squarer A, et al. Podocytopenia and disease severity in IgA nephropathy. Kidney international. 2002;61(4):1475-85.

36. Kretzler M. Role of podocytes in focal sclerosis: defining the point of no return. Journal of the American Society of Nephrology : JASN. 2005;16(10):2830-2.

37. Kriz W, Shirato I, Nagata M, LeHir M, Lemley KV. The podocyte's response to stress: the enigma of foot process effacement. Am J Physiol-Renal. 2013;304(4):F333-F47.

38. Hodgin JB, Rasoulpour M, Markowitz GS, D'Agati VD. Very low birth weight is a risk factor for secondary focal segmental glomerulosclerosis. Clinical journal of the American Society of Nephrology : CJASN. 2009;4(1):71-6.

39. Ikezumi Y, Suzuki T, Karasawa T, Yamada T, Hasegawa H, Nishimura H, et al. Low birthweight and premature birth are risk factors for podocytopenia and focal segmental glomerulosclerosis. American journal of nephrology. 2013;38(2):149-57.

40. Gubhaju L, Sutherland MR, Yoder BA, Zulli A, Bertram JF, Black MJ. Is nephrogenesis affected by preterm birth? Studies in a non-human primate model. American journal of physiology Renal physiology. 2009;297(6):F1668-77.

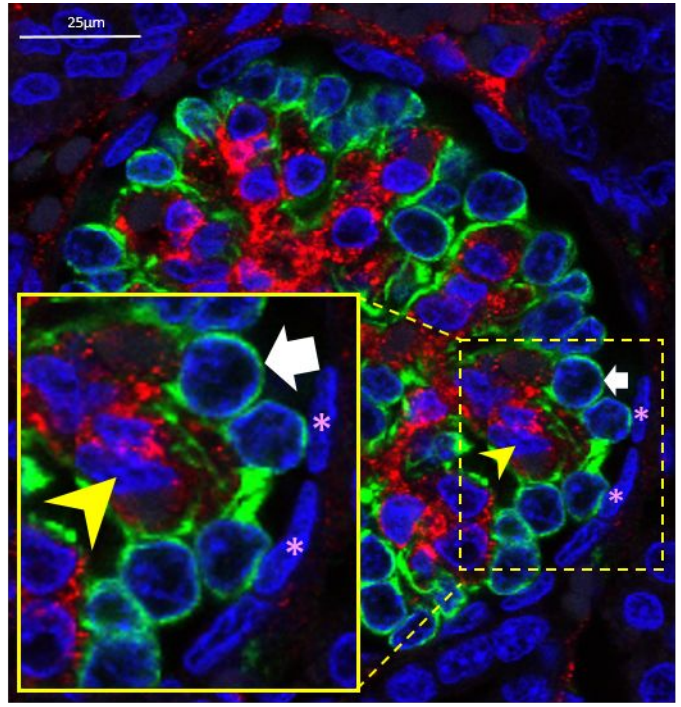

Figure 1: Representative immunolabelled glomerulus from the inner cortex of one of the term infants. Cell nuclei are stained with DAPI (blue). Podocytes were identified by WT-1 cytoplasmic immunofluorescence (white arrows). For non-podocyte cells, endothelial cells were immunostained with von Willebrand factor (red; yellow arrow) and mesangial cells (no immunostaining) were also assessed. Parietal epithelial cells on the Bowman's capsule (pink asterisk) are also seen. Scale bar = 25 µm.

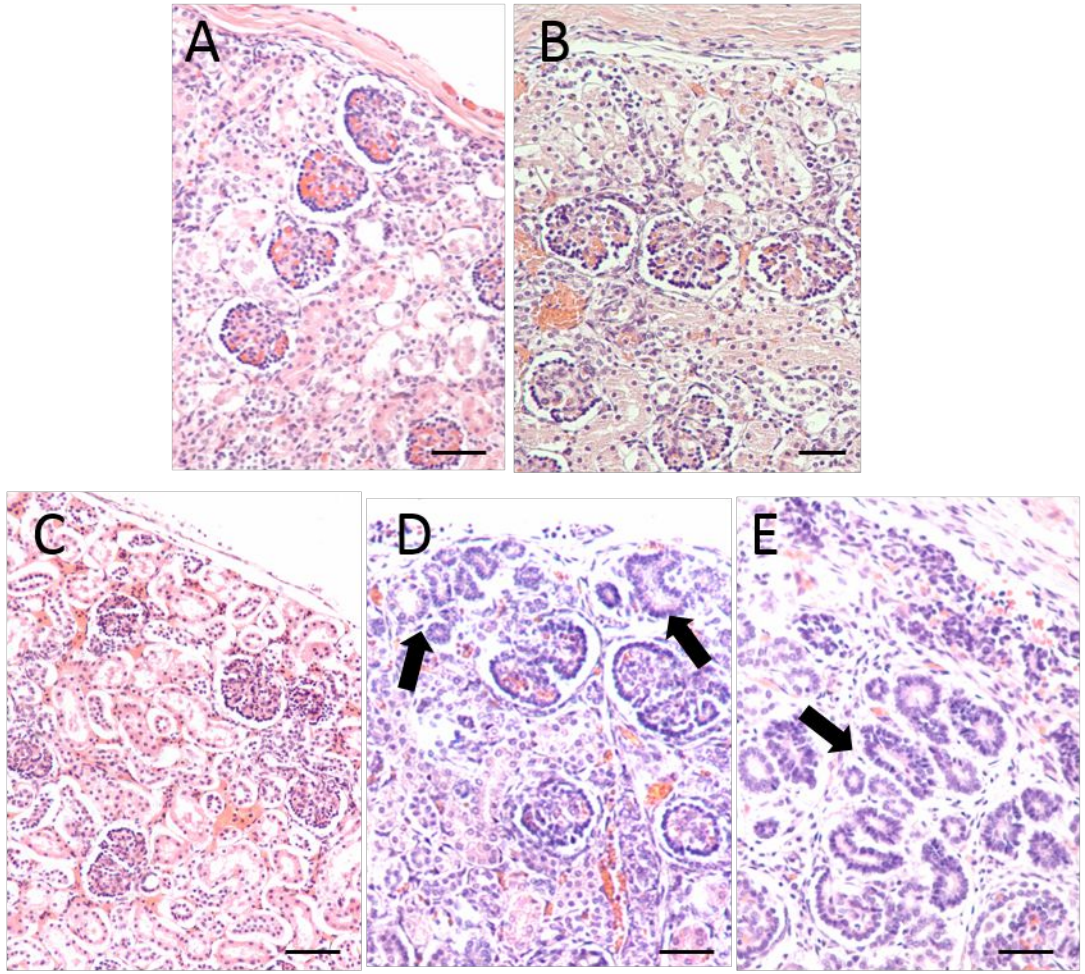

Figure 2. Light micrographs of the outer renal cortex from : (A) Infant 1; a term stillborn infant at 39 weeks of gestational age (nephrogenesis has ceased); (B) Infant 2; a term stillborn infant at 39 weeks gestational age (nephrogenesis has ceased); (C) Infant 3; a preterm infant born at 34 weeks' gestational age and died at 40 weeks' post-conceptional age (term equivalent age; nephrogenesis has ceased); (D) Infant 4; a preterm infant born at 32 weeks' gestational age and died at 38 weeks' post conceptional age (term equivalent age; ongoing nephrogenesis (arrows)); (E) Infant 5; a preterm infant born at 27 weeks' gestational age and died 32 weeks' post-conceptional age (ongoing nephrogenesis (arrows)). Kidneys sectioned at 5 µm and stained with Haematoxylin and Eosin. Scale bar = 50 µm.

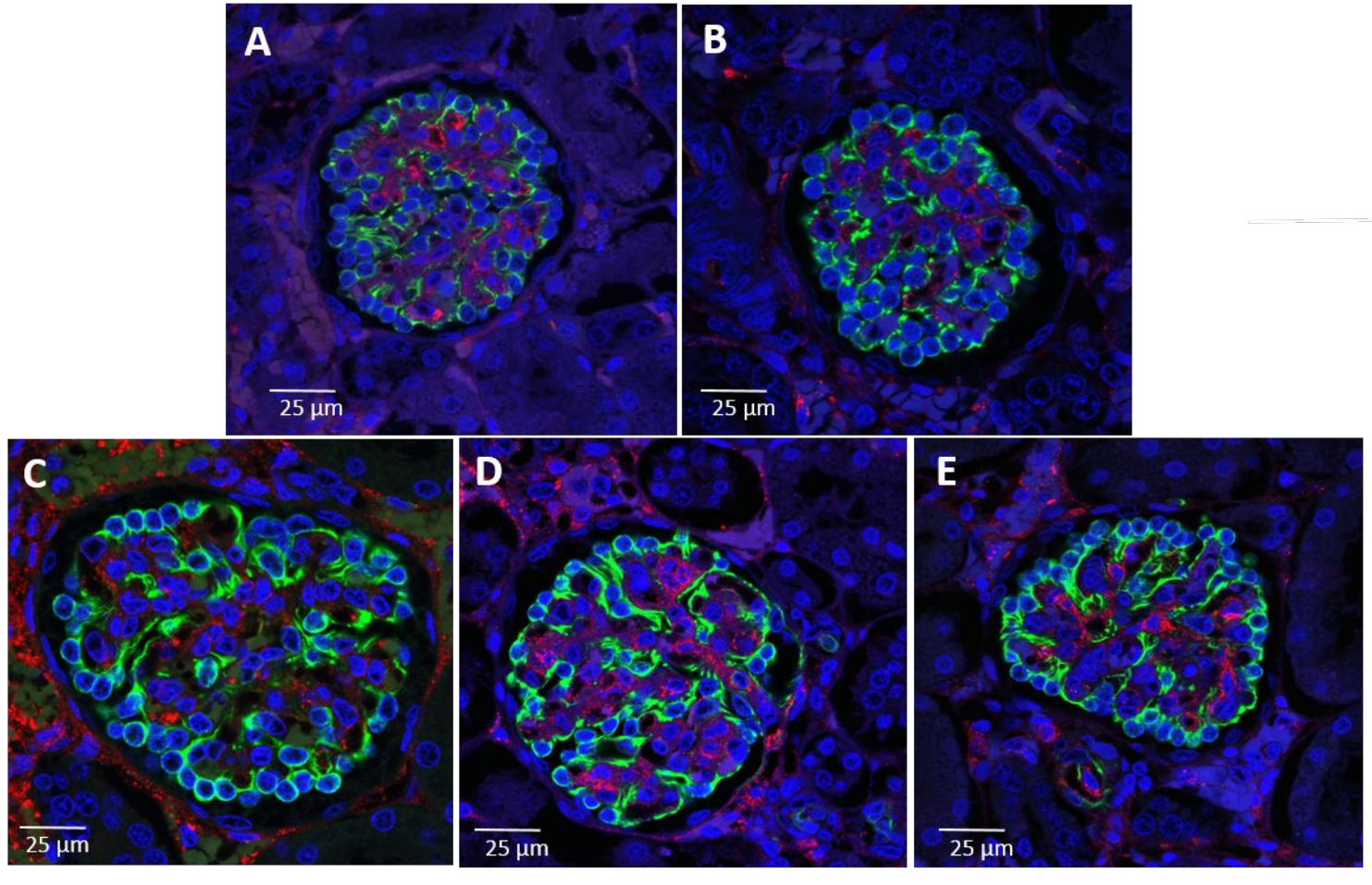

Figure 3: Representative immunolabelled glomeruli from: (A) Infant 1; a term stillborn infant at 39 weeks' gestational age; (B) Infant 2; a term stillborn infant at 39 weeks' gestational age; (C) Infant 3; a preterm infant born at 34 weeks' gestational age and died at 40 weeks' post-conceptional age (term equivalent age); (D) Infant 4; a preterm infant born at 32 weeks' gestational age and died at 38 weeks' post-conceptional age (term equivalent age); (E) Infant 5; a preterm infant born at 27 weeks' gestational age and died at 32 weeks' post-conceptional age. Green, WT-1 immunostaining in podocyte cytoplasm; red, vWF immunostaining in endothelial cells; blue, DAPI staining in cell nuclei. Scale bars = 25µm.

Infant	Gestational age at birth (weeks)	Postnatal age (days)	Post-conceptional age at autopsy (weeks)	Glomerulus #	Number of podocytes per glomerulus	Number of non-podocyte cells per glomerulus	Number of parietal epithelial cells per glomerulus	Glomerular volume (x 10^6 μm^3)	Podocyte density (n/10^6 μm^3)
GLOMERULUS OF TERM INFANTS									
1	39	0	39	1	289	139	53	0.31	941
				2	327	162	66	0.40	825
				3	344	196	56	0.43	805
				4	239	99	48	0.27	884
				5	288	165	54	0.36	794
				6	291	86	39	0.30	976
	Mean ± SD				**296 ± 36**	**141 ± 42**	**53 ± 9**	**0.34 ± 0.06**	**871 ± 75**
2	39	0	39	1	378	293	99	0.27	1410
				2	215	142	51	0.25	867
				3	394	210	67	0.35	1133
				4	320	257	39	0.21	1553
				5	363	185	76	0.18	1974
				6	459	226	93	0.35	1311
	Mean ± SD				**355 ± 82**	**219 ± 53**	**71 ± 24**	**0.27 ± 0.07**	**1375 ± 378**
GLOMERULUS OF PRETERM INFANTS									
3	34	41	40	1	421	306	74	0.54	783
				2	338	298	49	0.36	948
				3	355	244	71	0.45	786
				4	286	159	48	0.37	769
				5	309	200	63	0.34	914
				6	318	238	43	0.40	802
	Mean ± SD				**338 ± 47**	**241 ± 56**	**58 ± 13**	**0.41 ± 0.07**	**834 ± 77**
4	32	42	38	1	400	228	78	0.50	799
				2	363	163	62	0.45	815
				3	380	244	65	0.51	750
				4	399	261	58	0.54	742
				5	356	237	93	0.49	724
				6	376	230	83	0.39	956
	Mean ± SD				**379 ± 18**	**227 ± 34**	**73 ± 14**	**0.48 ± 0.05**	**789 ± 85**
5	27	34	32	1	338	192	76	0.28	1192
				2	338	220	95	0.46	737
				3	214	143	66	0.29	726
				4	260	172	70	0.29	889
				5	235	124	49	0.27	863
				6	214	152	56	0.25	855
	Mean ± SD				**267 ± 58**	**167 ± 35**	**69 ± 16**	**0.31 ± 0.08**	**877 ± 169**

Chapter Five:
INTRAUTERINE GROWTH RESTRICTION: IMPACT ON NEPHROGENESIS AND ON RENAL FUNCTION IN THE PRETERM NEONATE

CHAPTER FIVE DECLARATION

Declaration by candidate
[It is to be noted that I have used my married surname (Ryan) in this manuscript]

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

Nature of contribution	Extent of contribution (%)
I conducted the majority of the experimental work, infant recruitment, urine collection and protein analysis for renal function studies, data collection and analyses and wrote the manuscript.	80%

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

Name	Nature of contribution	Extent of contribution (%) for student co-authors only	Co-author(s), Monash student Y/N
Megan Sutherland, Peter Coombs, Tracey Flores, Lynette Moore, Alison Kent, Jane Dahlstrom, Shanti Diwarkarla, Flora Wong, Wendy Hoy, Rosemary Horne, M. Jane Black	Involved in the design of experiments, obtained funding, performed ultrasound scans, assisted with some experimental analysis and assisted in editing the manuscript		N

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

Candidate's Signature	[redacted]	Date *21.9.16*
Main Supervisor's Signature	[redacted]	Date *21.9.16*

INTRAUTERINE GROWTH RESTRICTION: IMPACT ON NEPHROGENESIS AND ON RENAL FUNCTION IN THE PRETERM NEONATE

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ABSTRACT

Intrauterine growth restriction (IUGR), defined as growth below the 10th percentile for gestational age, is a common antecedent of preterm birth, and is often a co-morbidity in preterm neonates. To date, there have been very few studies that have looked at the effect of IUGR on nephrogenesis in the developing human kidney. Therefore, the aims of this study were to further characterise the effects of IUGR on nephrogenesis in the developing human kidney from mid gestation through to term and secondly, to assess the impact on renal growth and function in the first month of life when IUGR is combined with preterm birth. In order to address the first aim, kidney samples were obtained at autopsy from 90 infants that died acutely *in utero* or died within 24 hours after birth. Using image analysis, nephrogenic zone width, the number of glomerular generations and renal corpuscle cross-sectional area was examined. There was marked attenuation of kidney growth during late gestation, leading to a reduction in the number of glomerular generations formed within the kidneys. Furthermore, the growth of glomeruli during late gestation differed in the IUGR and non-IUGR kidneys. To address the second aim, 40 preterm infants were recruited, and assessed for kidney size (measured using ultrasound) and protein excretion within the first month of life. Spot urine samples were collected on days 8, 15, 22 and 29 after birth, and were used to measure levels of urinary total protein (g/L), albumin (mg/L) and β2-microglobulin (mg/L), which were all corrected for urine creatinine (µmol/L) levels. When combined with preterm birth, IUGR led to reduced kidney size at day 29 of life, and greater impairment of renal function, with marked adverse effects on renal tubular function; as evidenced by a significantly elevated excretion of ß2-microglobulin in the IUGR infants at days 8, 15 and 22 of life. Overall, the findings highlight the importance to renal development and postnatal renal function of maintaining optimal body growth during late gestation.

INTRODUCTION

It is well-recognised that intrauterine growth restriction (IUGR), defined as growth below the 10th percentile for gestational age, can adversely impact renal development and nephrogenesis in the developing fetal kidneys (Hinchliffe et al., 1992, Manalich et al., 2000, Rodriguez-Soriano et al., 2005, Moritz et al., 2009, Wang et al., 2015). Importantly, numerous experimental studies have shown that IUGR leads to a reduction in kidney size at birth and this is accompanied by reduced nephron endowment (Lucas et al., 1997, Bassan et al., 2000, Woods et al., 2004, Almeida and Mandarim-de-Lacerda, 2005, Schreuder et al., 2006, Zohdi et al., 2007). Studies in autopsied adult human kidneys have also shown a direct relationship between birth weight and nephron number; for each 1kg increase in birth weight it has been reported that there is an increase of approximately 250,000 nephrons (Hughson et al., 2003). The observed reduction in nephron endowment following IUGR has important repercussions for lifelong renal functional reserve, given that nephrogenesis (the formation of nephrons) only occurs in early development and is normally complete by birth in the term-born infant. In general, nephrogenesis is completed late in gestation (from 32 to 36 weeks' gestation) (Hinchliffe et al., 1991, Sutherland et al., 2011), with no new nephrons formed after this time for the lifetime of the individual (Osathanondh and Potter, 1963, Hinchliffe et al., 1991). To date there have been very few studies that have examined the effect of IUGR on nephrogenesis in the developing human kidney; these analyses have been conducted in only a small number of infants who died at the time of birth or within one year of birth (Hinchliffe et al., 1992, Manalich et al., 2000). The first of these studies, conducted by Hinchliffe et al in 1992, investigated nephron endowment in 14 IUGR infants (6 were still born infants and the other 8 infants died before 1-year postnatal age). When compared with non-IUGR infants (11 stillborn infants and 7 infants at 1 year of age) there was a significant reduction in the number of glomeruli within

the kidneys of the IUGR infants. In another study of 35 deceased infants, there was a positive correlation between weight at birth and the number of glomeruli with a 20% reduction in nephron number in infants of low birth weight (Manalich et al., 2000).

IUGR is a common antecedent of preterm birth (birth before 37 completed weeks of gestational age), and hence IUGR is often a co-morbidity in preterm neonates. The kidneys of the preterm newborn are particularly vulnerable to injury in the neonatal period because of the increased functional demands and increased renal blood flow following birth and the exposure of nephrotoxic medications used in their neonatal care. Importantly nephrogenesis does continue after birth in infants born prematurely; however, there is induction of renal and glomerular hypertrophy and accelerated postnatal maturation of glomeruli (Sutherland et al., 2011). Because of their relative renal immaturity, preterm infants experience a low glomerular filtration rate (GFR) and high sodium excretion, with levels dependent on gestational and postnatal age (Gubhaju et al., 2014, Gallini et al., 2000, Thayyil et al., 2008). Importantly, high levels of urine protein excretion occur after preterm birth (Gubhaju et al., 2014, Awad et al., 2002), and we have previously shown that this can persist postnatally (Gubhaju et al., 2014). There have been a multitude of epidemiological studies linking low birth weight (which can originate from IUGR and/or preterm birth) with adult renal disease; a meta-analysis of 31 of these studies reported a 70% increased risk of developing chronic renal disease in subjects born of low birth weight (White et al., 2009). Hence in order to improve long-term adult renal health, it is imperative to gain an understanding of how IUGR affects the developing kidney and the combined effects of IUGR and preterm birth on renal development and function in the neonatal period. Such findings are of utmost clinical importance; given that the neonatal period provides a critical window for intervention in preterm neonates where nephrogenesis is ongoing after birth.

Hence the aims of this study were to characterise the effects of IUGR on nephrogenesis in the developing human kidney from mid gestation through to term (the gestational period when the majority of nephrons are formed) and secondly, to assess the impact on renal growth and function in the first month of life when IUGR is combined with preterm birth. In order to address the first aim, we conducted a study of renal growth and structure in kidneys obtained at autopsy from stillborn IUGR and non-IUGR infants. All infants died acutely *in utero* and had no overt congenital abnormalities. To address the second aim, we conducted a study of kidney size and protein excretion in newborn IUGR and appropriately grown for gestational age preterm infants within the first month of life.

METHODS AND MATERIALS

To address the aims, analyses were initially conducted in archived human kidneys (Study 1) to examine the effect of IUGR on late gestational renal development. The second study (Study 2) examined the effect of IUGR on kidney size and renal function in newborn preterm IUGR and non-IUGR neonates.

Study 1: Effect of IUGR on renal development and nephrogenesis Archived kidneys collected at autopsy:

In this retrospective study, 90 archived fetal kidneys were obtained from The Women's and Children's Hospital in North Adelaide, South Australia, and the Canberra Hospital in the Australian Capital Territory. The kidneys were collected at autopsy from IUGR and non-IUGR babies that died suddenly *in utero*, or died within 24 hours of birth; ranging in age from 20 to 41 weeks of gestation (IUGR n = 39/90 [43.3%]; non-IUGR n = 51/90 [56.7%]). Autopsies were performed between 1996 -2013. The infants were classified at autopsy as IUGR (growth below the 10th percentile for gestational age), based on birth weight and ponderal index. Infants were excluded from the study if there was evidence of any congenital abnormalities, cardiovascular or renal complications, or exposure to chorioamnionitis, or

in utero. Kidneys were also excluded if they were moderately or severely macerated.

Clinical histories were obtained from the autopsy reports, with gestational age at birth, birth and autopsy weights, head circumference, crown-ump length, abdominal circumference and kidney weights recorded.

Ethical approval for this study 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. Written parental consent for the study of tissues for scientific purposes was obtained or consent was given for post mortem and to retain tissue for research purposes.

Kidneys collected at autopsy were weighed, and only the left kidney was cut in half in the longitudinal plane and further cut along a transvers plane and immersion-fixed in 10% (v/v) buffered formalin to be further analysed. The fixed kidney portions were embedded in paraffin b blocks, sectioned at 5µm and stained with haematoxylin and eosin. Researchers were blinded to the gestational ages and study groups during the analyses.

Assessment of nephrogenesis

The outer renal cortex was morphologically examined to determine whether nephrogenesis was ongoing at the time of autopsy. Nephrogenesis was considered to be ongoing when there was a visible nephrogenic zone in the outer renal cortex, as evidenced by the presence of metanephric mesenchyme and immature nephrons in the form of comma and S-shaped bodies. Nephrogenesis was considered to have ceased when there was no evidence of immature developing glomeruli within the outer renal cortex, with only mature glomeruli (glomeruli with a well-defined glomerular tuft and distinct Bowman's capsule) located in the outer renal cortex.

Nephrogenic Zone Width

The width of the nephrogenic zone was measured in kidneys exhibiting ongoing nephrogenesis, using image analysis software (Image Pro Plus v. 6.0 for Windows, Media Cybernetics; Silver Spring, MD, USA). Each kidney section was viewed at 200x magnification, and the width of the nephrogenic zone (delineated as the region in the outer renal cortex exhibiting immature glomerular structures and metanephric mesenchyme) was measured in four randomly sampled regions of the cortex, with average nephrogenic zone width per kidney subsequently determined.

Number of glomerular generations

In histological sections, spanning from the medulla to the outer renal capsule, the number of glomerular generations formed within the kidneys was assessed. Using a medullary ray glomerular counting method (Hinchliffe et al., 1991, Sutherland et al., 2011), the number of glomeruli were counted along 5 clearly defined medullary rays per kidney and the average number of glomerular generations for each kidney was then determined.

Assessment of renal corpuscle cross sectional area

Given that the kidneys were immersion-fixed, and therefore it was likely that the glomerular capillaries would not be patent, it was considered that renal corpuscle cross-sectional area would provide a more accurate assessment of glomerular size rather than glomerular cross-sectional area. Renal corpuscle cross-sectional area of 100 glomeruli per kidney was measured by tracing the inner boundary of Bowman's capsule, using image analysis software (Image Pro Plus v. 6.0 for Windows, Media Cybernetics; Silver Spring, MD, USA). Glomeruli were sampled in kidney sections using a systematic sampling method at 400x magnification. At each field of view only one renal corpuscle was measured; when more than one renal corpuscle was present in the field of view the renal corpuscle to be measured was selected in an unbiased manner according to the method of Nyengaard and Marcussen (1993).

Statistical analysis

All data were analysed using GraphPad Prism v5.03 for windows (GraphPad Software, San Diego, California). Linear regression analyses were undertaken to determine correlations between the indices of fetal growth (birth weight and kidney weight) and renal morphology (nephrogenic zone width, glomerular generation number, renal corpuscle cross-sectional area) *versus* gestational age. The slope and y-intercept of the regression lines were then compared between IUGR and non-IUGR groups using analysis of covariance (ANCOVA). In recent studies in our laboratory (unpublished findings) we have observed sexual dimorphism in the growth of glomeruli, as assessed by renal corpuscle area, in the latter half of gestation (20 weeks of gestation until term) and this was also observed in the current study; hence, renal corpuscle area was analysed and reported separately for each sex. As there were no statistically significant differences between the sexes in any of the other parameters that were examined, the male and female data were subsequently pooled and the pooled data are reported in the figures. Final number of glomerular generations in the IUGR and non-IUGR groups was assessed using a two-tailed Student's t-test, and results are reported as the mean ± SD. The level of statistical significance was accepted at $P < 0.05$.

Study 2: Assessment of renal function during the first month of life

In IUGR and non-IUGR infants born very preterm (< 32 weeks' of gestation) and moderately preterm (32-36 weeks' gestation), kidney growth and renal function was assessed over the first month of life.

Infants studied:

Preterm IUGR and non-IUGR neonates (<37 weeks of gestation) were recruited from the neonatal intensive care and special care units at Monash Medical Centre from 2012 to 2015. Over the study period, 40 infants were recruited (3 of which withdrew prior to the completion

of the study period). The infants were classified at birth as IUGR or non-IUGR based on birth weight for gestational age and ponderal index. The infants were categorised according to their gestational ages at birth into; very preterm (there were 17 infants from 26 to 31 weeks' of gestation; IUGR n = 6/17 [35.3%] and non-IUGR n = 11/17 [64.7]%); and moderately preterm infants (there were 20 infants from 32 to 36 weeks' of gestation; IUGR n = 10/20 [50%] and non-IUGR n = 10/20 [50%]). Infants were not recruited for the study if there was evidence of congenital abnormalities or cardiovascular or renal complications.

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

Infant recruitment and urine collection

Mothers that had delivered preterm were briefed about the study 48 hours after giving birth in order to recruit the infants into the study. If consent was given, spot urine samples, collected via urine collection bags were collected on days 8, 15, 22 and 29 after birth. For some infants, it was not feasible to collect spot urine samples on all of the study collection days; for example, urine collection bags could not be placed on some very preterm infants, due to their delicate skin. In these cases, cotton balls were placed in the diaper of the infant and the urinary proteins extracted from the cotton balls using an established protocol (Fell et al., 1997). In addition, over the course of the study period, it was not always possible to collect the data for every infant at every time point. This explains the variability in the number of subjects (n values) within the study groups, in the different renal assessments presented in the results section.

Spot urine samples were used to measure levels of urinary total protein (g/L), albumin (mg/L), and β2-microglobulin (mg/L), which were all corrected for urine creatinine (μmol/L) levels. Urine analyses were performed by the Monash Health Pathology Department (Southern Cross Pathology; Clayton, Australia). Urine creatinine was measured with a modified Jaffe reaction

colorimetry method, using a Beckman Coulter SYNCHRON LX20PRO® system, with reagents and calibrators supplied by Beckman Diagnostics (Sydney, Australia). Urine total protein (UTP), albumin, and β2-microglobulin were measured using nephelometric technology on a Beckman Coulter immunochemistry system, with reagents and calibrators supplied by Beckman Diagnostics (UTP and albumin; Beckman Diagnostics; Sydney, Australia) and DakoCytomation (β2-microglobulin; DakoCytomation; Glostrup, Denmark) (Gubhaju et al., 2014).

Assessment of kidney growth over the first month of life

Renal ultrasound scans were performed by trained sonographers at 8 and 29 days after birth; the scans at 29 days were not performed in all infants, especially when the infants had already been discharged from hospital.

Kidney size and cortical thickness, were measured with a high resolution linear transducer using a strict measurement protocol. The infants were positioned in a prone or lateral decubitus position. The transducer was placed posteriorly adjacent to the spine. Three separate images in the longitudinal plane were recorded with renal length measured from the maximum distance between the outer capsule margins (Figure 1A). These length measurements were then averaged to determine the length per kidney. The same longitudinal images were then used to measure the cortical thickness. A medullary pyramid was selected in the middle of the kidney that was oriented orthogonal to the renal length. A measurement through the middle of this pyramid was recorded from the outer edge of the renal capsule to the apex of the medullary pyramid (see arrow - Figure 1A). If there was a thin layer of sinus fat obscuring the papillary margin, this was included in the measurement. The transverse measurements were then performed by rotating the transducer 90 degrees and measuring the height and width of the kidneys from the maximum capsular margins (Figure 1B). This was performed three times,

and an average of the measurements was calculated. The renal length, height, and width measurements were then used to calculate kidney volume using the following formula:

Kidney volume = kidney length x kidney width x kidney depth x 0.523 (Kim et al., 2013, Weitz et al., 2013).

Statistical analysis

Statistical analyses were performed using GrapPad Prism v6.04 for Windows and Intercooled Stata v14 for Windows. Data are presented as medians [Inter Quartile Range]. Statistical significance was accepted at the level of $P < 0.05$.

Birth characteristics (gestational age, birth weight, length, head circumference), and urine protein levels (urine total protein-, albumin-, and β2-microglobulin-to-creatinine ratios), were compared among groups using two-way ANOVA, followed by a Bonferroni post hoc test.

The factors assessed in all of these analyses were: degree of prematurity (P_{GA}), effect of IUGR (P_{IUGR}) and their interaction ($P_{GA \times IUGR}$).

RESULTS

Study 1

Effects of IUGR on body growth during late gestational development

Average body weights of the IUGR and non-IUGR fetuses at the gestational time-points studied are shown in Figure 2A. The body weights represent the weights of the infants at delivery (IUGR n = 35 and non-IUGR n = 42). Given that body weight is affected by the period of time between delivery and autopsy, we have only presented the body weights at the time of birth; birth weights were not available for all infants. From 20 weeks of gestation until term, body growth increased with increasing gestational age in both IUGR and non-IUGR fetuses; however, weight gain over the gestational period was significantly reduced in IUGR compared to non-IUGR fetuses ($P < 0.0001$; Figure 2A).

In both IUGR and non-IUGR fetuses, head circumference (Figure 2B), and crown-rump length (Figure 2D) significantly increased over the gestational period, but the IUGR fetuses remained markedly smaller in both crown rump length ($P < 0.0001$) and head circumference ($P = 0.0005$) when compared to non-IUGR fetuses. Overall, there was a strong linear relationship between head circumference and body weight within both the IUGR and non-IUGR groups (Figure 2C). Importantly, however, head circumference relative to body weight differed between groups ($P < 0.0001$), with evidence of asymmetric growth in the IUGR fetuses compared to non-IUGR fetuses, particularly those > 1000g in body weight (Figure 2C).

Effects of IUGR on kidney growth during gestational development

In accordance with body growth from 20 weeks of gestation until term, there was a strong positive correlation between kidney weight and gestational age in both IUGR and non-IUGR fetuses (Figure 3A). The gain in kidney weight with increasing gestation, however, was significantly less in the IUGR fetuses compared to the non-IUGR fetuses ($P = 0.0002$; Figure 3A). When kidney weight was adjusted for body weight, there was no correlation with gestational age in either the IUGR or non-IUGR fetuses (Figure 3B), indicative of kidney growth being proportional to body growth over the gestational period in both groups.

Assessment of nephrogenesis during gestational development

Nephrogenic zone width

Of all of the kidneys studied, nephrogenesis was ongoing at the time of autopsy in 30/39 [76.9%] of the IUGR infants and 46/51 [90.2%] of the non-IUGR infants, with evidence of metanephric mesenchyme and developing glomeruli in the form of comma and S-shaped bodies in the outer renal cortex. In both IUGR and non-IUGR fetuses, the nephrogenic zone width decreased with gestational age (Figure 4A); overall, there was no significant difference in the change in nephrogenic zone width over the gestational period between the IUGR and non-IUGR fetuses.

In 17 fetuses that were born prior to term (6/39 [15.4%] IUGR and 10/51 [19.6%] non-IUGR), there was no evidence of a nephrogenic zone, thus indicating that nephrogenesis was complete at the time of autopsy. As expected, in most of the term kidneys analysed (26 term kidneys: 3/26 [11.5%] IUGR and 23/26 [88.5%] non-IUGR), nephrogenesis was complete at the time of autopsy with the exception of one non-IUGR term infant (37 weeks' gestation) that demonstrated ongoing nephrogenesis.

Timing of the cessation of nephrogenesis

Overall, nephrogenesis was ongoing in the kidneys of all infants ranging in age from 20-32 gestational weeks (Table 1). Interestingly, after 32 weeks of gestation there was a wide range in the timing of the cessation of nephrogenesis in the non-IUGR kidneys, where nephrogenesis had ceased in an non-IUGR infant as early as 33 weeks' gestation whereas in another non-IUGR infant nephrogenesis was still ongoing at 37 weeks of gestation. In all IUGR kidneys studied, nephrogenesis was complete after 35 weeks' gestation (Table 1). In the non-IUGR fetuses, nephrogenesis was ongoing after 35 weeks' gestation in some but not all kidneys; at 36 weeks' gestation (1 out of 5 kidneys) and 37 weeks' gestation (1 out of 5 kidneys).

Glomerular generations

From 20 weeks of gestation, there was a strong positive linear correlation between gestational age and the number of glomerular generations in kidneys from both IUGR and non-IUGR infants (Figure 4B). Notably, however, the number of glomerular generations formed within the renal cortex was significantly decreased in the IUGR fetuses compared to non-IUGR fetuses ($P = 0.0001$; Figure 4B). In addition, in both IUGR and non-IUGR fetuses, there was a strong linear relationship between the number of glomerular generations and kidney weight (Figure 4C), and body weight (Figure 4D). Of note, in comparison to non-IUGR fetuses, the number of glomerular generations formed in the kidneys of the IUGR fetuses increased more rapidly in relation to both kidney weight ($P = 0.0088$; Figure 4C) and body weight ($P = 0.0013$; Figure 4D).

In fetuses where nephrogenesis had ceased at the time of autopsy (fetuses ranging in age from 33 weeks to 41 weeks' gestation; Table 1), the final number of glomerular generations varied from 8 -12 glomerular generations in the non-IUGR fetuses and 8 -11 glomerular generations in the IUGR fetuses. The average number of glomerular generations formed within the kidneys was significantly less in the IUGR fetuses (9 ± 0.3) than the non-IUGR fetuses (10 ± 0.2; P = 0.025).

Renal corpuscle cross sectional area

The growth of glomeruli, from 20 weeks of gestation until term, was different in IUGR kidneys compared to non-IUGR kidneys. In non-IUGR kidneys there was a significant increase in renal corpuscle cross-sectional area with increasing gestational age (Figure 5A), kidney weight (Figure 5B), body weight (Figure 5C), and number of glomerular generations (Figure 5D). Conversely, in the IUGR fetuses, renal corpuscle cross-sectional area significantly decreased with increasing gestational age (Figure 5A); the difference between groups was particularly evident in fetuses > 26 weeks of gestation. There was no significant correlation between renal corpuscle cross-sectional area and kidney weight, body weight, or the number of glomerular generations in IUGR fetuses. Importantly, however, there were significant differences in the slopes of the regression lines between groups, whereby renal corpuscle size was not augmented in relation to increasing body or kidney growth in the IUGR fetuses as occurred in the non-IUGR fetuses (Figure 5B-D).

Given that in a previous study in our laboratory we found differences in the growth of glomeruli between male and female fetuses over gestation (we separately analysed renal corpuscle cross-sectional area for each sex, unpublished findings). Female non-IUGR fetuses showed a strong relationship between renal corpuscle cross-sectional area and gestational age (Figure 5E), whereas, in the kidneys of non-IUGR male fetuses this relationship was not evident (Figure 5F).

In contrast, in the male IUGR fetuses, renal corpuscle cross-sectional area significantly decreased with gestational age (Figure 5F); this relationship was not evident in the IUGR female fetuses where renal corpuscle cross-sectional area remained constant throughout gestation (Figure 5E). Overall, kidneys of both IUGR males and IUGR females exhibited a decrease in renal corpuscle cross-sectional area with increasing gestational age when compared to non-IUGR males and females (P = 0.033; P = 0.046 respectively).

Study 2

Growth measurements at birth

Body weight, head circumference and body length at the time of birth, for all infants studied, are reported in Table 2. Body weight (Figure 6A), head circumference (Figure 6B) and body length (Figure 6C) increased significantly with gestational age at birth in both IUGR and non-IUGR infants; however, head circumference (Figure 6B) and body length (Figure 6C) were both significantly reduced in the IUGR infants when compared with their non-IUGR age-matched counterparts. The rate of growth in body weight with increasing gestational age was also significantly reduced in the IUGR infants compared to the non-IUGR group (P = 0.018; Figure 6A).

There were a number of twin infants in both the IUGR and non-IUGR preterm groups, but all term infants were singletons (Table 2). Notably, a high proportion of the infants born moderately preterm were males, whereas the majority of the preterm infants in the very preterm groups were females (Table 2).

Kidney growth in the first month of life

Effect of gestational age at birth and IUGR on postnatal kidney growth

The right and left kidneys were measured using ultrasonography at day 8 and 29 of life, and the measurements are shown in Table 3. At postnatal day 8, kidney length, width, cortical depth

and kidney volume were significantly increased in the right kidney relative to gestational age at birth. In the left kidney, a significant increase relative to gestational age at birth was observed in relation to kidney length and depth, with a trend for kidney volume (P = 0.056; Table 3) to also be increased. At day 29 of life, the significant differences in kidney length, width, depth and volume in relation to gestational age at birth remained in the right kidney, whereas in the left kidney there was a significant effect of gestational age at birth only in relation to kidney length and volume.

Notably, there were no detectable differences in any of the kidney measurements between the IUGR and non-IUGR infants at day 8 of life in either the right or left kidney, with the exception of kidney depth which was significantly reduced in the very preterm IUGR infants (Table 3). At day 29, however, there was a significant reduction in both kidney width and volume as a result of IUGR in the preterm infants (Table 3), but this effect of IUGR on kidney growth was only observed in right kidneys.

Renal function over the first month of life

At postnatal days 8, 15, 22 and 29 of life, the effects of gestational age at birth and of IUGR on renal function, glomerular function and tubular function were assessed by measuring urinary protein, albumin and β2-microglobulin levels, respectively, relative to urine creatinine levels, and the findings are shown in Figure 7A-L.

Overall, gestational age at delivery (very preterm compared to moderately preterm) had no effect on total urinary protein/creatinine levels at any of the time points measured (Figure 7A-D).

At day 8, there were significant reductions in the levels of urinary albumin/creatinine (Figure 7B) and β2-microglobulin/creatinine (Figure 7I) with increasing gestational age at birth; that is, between the infants born very preterm with those born moderately preterm. In the subsequent

weeks at days 15, 22 and 29 there was no significant effect of gestational age at birth on any of the parameters measured (Figure 7E - K), except β2-microglobulin at day 29 after birth, where the levels of β2-microglobulin/creatinine were significantly elevated in the infants born very preterm compared to those born moderately preterm (Figure 7L).

Notably, there was a significant increase in the albumin/creatinine (Figure 7E) and β2-microglobulin/creatinine (Figure 7I) levels in the preterm IUGR infants at day 8 of life, when compared to non-IUGR preterm infants. Significantly elevated levels of β2-microglobulin/creatinine in the preterm IUGR infants persisted at days 15 and 22 of life, but were not different to the non-IUGR infants at day 29 (Figure 7J-L). Overall, grossly abnormal function was observed in only 5 of the urinary measurements over the study period; these abnormally high values, defined as 1.5 times above the 75% percentile, are shown on the graphs (Figure 7A, B, D, J); the majority of these infants were non-IUGR. In particular, there was overt proteinuria (UTP ≥ 500 mg/l) in four of these infants, only one of whom was IUGR.

DISCUSSION

IUGR is a common co-morbidity of preterm birth. The findings of these studies highlight the vulnerability of the developing kidney to late gestational IUGR, with marked attenuation of kidney growth during this period, leading to a reduction in the number of glomerular generations formed within the kidneys. When combined with preterm birth, IUGR leads to greater impairment of renal function in the preterm neonate, with marked adverse effects on renal tubular function.

Our studies of renal development in the human kidney, showed that kidney growth during the second half of gestation is attenuated in IUGR infants, with kidney weights in both IUGR and non-IUGR directly proportional to body weight. The growth of glomeruli during this gestational period, as assessed by renal corpuscle cross-sectional area, differed in the IUGR and non-IUGR

kidneys and this was also influenced by the sex of the fetus. Overall, the number of glomerular generations formed within the kidneys over the second half of gestation was less in IUGR fetuses, which supports the concept that there are fewer nephrons formed in the kidneys of IUGR infants. This in turn highlights the importance of maintaining optimal body growth during late gestation in order to achieve an adequate nephron endowment.

During the second half of gestation, there was a marked reduction in kidney weight in the IUGR infants, with kidney weight directly proportional to body weight. In contrast, head circumference was not significantly different between the IUGR and non-IUGR fetuses, over the gestational period; so although body growth was markedly attenuated in IUGR infants the growth of the head was not affected and this is indicative of asymmetrical growth. Indeed, it is well described when growth restriction occurs late in gestation that there is often preferential blood flow to the brain, termed 'brain sparing', as an adaptive physiological response of the fetus in an attempt to maintain normal growth of the fetal brain (Barker, 1995). This redistribution of fetal blood flow occurs at the expense of the visceral organs, such as the kidneys (Behrman et al., 1970).

In accordance with our findings, previous studies of fetal growth conducted in pregnant women using ultrasonography, have reported reduced abdominal circumference, smaller biparietal diameter, reduced femur length and a 31% decrease in renal volume in IUGR fetuses compared to non-IUGR fetuses (Silver et al., 2003). Similarly, fetal ultrasound measurements from different frames of the anterior- posterior, transverse and circumference of both kidneys (Konje et al., 1996), have shown that IUGR fetuses exhibit a slowing of kidney growth between 26 and 34 weeks' gestation compared to non-IUGR fetuses, resulting in overall reduced kidney size at term.

Given our findings and those of others (described above), that kidney growth is reduced in IUGR infants relative to non-IUGR infants, it was an unexpected finding in our neonatal studies that no differences were detected in renal length, width, depth and volume or cortical depth, using ultrasound at day 8 of life, between the IUGR and appropriately grown infants that were born very and moderately preterm. The findings may relate to the fact that in preterm infants there is marked hypertrophy of the kidneys in the neonatal period (Rodriguez et al., 2004, Sutherland et al., 2011). Hence, the acute induction of renal hypertrophy as a result of preterm birth may be sufficient to mask any attenuation of kidney growth that may have been present at birth. Interestingly, at day 29, significant reductions in kidney width and kidney volume were apparent in the infants born IUGR, relative to the infants born non-IUGR, but these effects were only observed in the right kidney. Hence, the findings suggest that the right kidney may be more vulnerable to IUGR. Indeed, there are a number of lines of evidence that suggest that there is enhanced growth in the left kidney relative to the right kidney; for example, the left kidney is bigger overall than the right kidney (Emamian et al., 1993), with an enhanced renal artery blood supply compared to the right (Satyapal et al., 2003). Retrospectively, we could not address this in our autopsy studies, as only the left kidneys were analysed.

In general, our studies in the autopsied kidneys, demonstrated that nephrogenesis was complete in the majority of infants at 36 weeks of gestation; however, there was variability in the timing of the cessation of nephrogenesis in both IUGR and non-IUGR infants. In the present study it was not possible to stereologically measure nephron number as we were unaware of the precise fraction of the kidney collected at autopsy. Hence, we measured the number of generations of glomeruli formed within the kidney as a proxy measure for nephron number. Importantly, we showed in the kidneys of infants where nephrogenesis had ceased, that the average number of glomerular generations in the IUGR infants was significantly less than in the

non-IUGR infants (9 versus 10 glomerular generations, respectively). This supports the concept that there is reduced nephron endowment in the kidneys of IUGR infants. Indeed, this has been widely described in animal models of IUGR (reviewed in (Zohdi et al., 2012). Previous studies in IUGR human infants have been limited and only conducted in a small number of infants. In those studies, there was also a reduction in the number of glomerular generations in the outer renal cortex (Hinchliffe et al., 1992, Manalich et al., 2000). Importantly our studies conducted in premature infants over the first month of life showed that both gestational age at birth and IUGR increase the vulnerability to renal injury during the first week of life. At day 8 of life, the infants born very preterm showed greater excretion of albumin and ß2-microglobulin (indicative of glomerular and tubular immaturity/injury, respectively) compared to those born moderately preterm, and that these effects were exacerbated in the infants born IUGR. After the first week of life, no differences in albumin excretion were observed between the very preterm and moderately preterm infants or between the IUGR and non-IUGR infants. Importantly, however, the IUGR infants continued to exhibit a greater degree of tubular injury over the first month of life compared to non-IUGR infants, as evidenced by a significantly elevated excretion of ß2-microglobulin in the IUGR infants at days 8, 15 and 22 of life.

It was not surprising in our studies that the excretion of ß2-microglobulin was affected by gestational age at birth. Indeed, it is well described that renal tubular maturation occurs late in gestation, with the majority of tubular growth occurring in the postnatal period (Fong et al., 2014). Likewise, the persistent elevation of ß2-microglobulin at 8,15 and 22 days after birth in the IUGR preterm infants likely reflects the adverse impact of IUGR on late gestational renal development, the critical period when tubular maturation occurs.

CONCLUSION

In conclusion, the findings of this study demonstrate that IUGR adversely impacts renal development and nephron endowment in human infants. Preterm birth causes further impairment of renal function to the preterm neonate, particularly in relation to renal tubular function. The findings highlight the importance of normal growth for optimal to renal development and postnatal renal function of maintaining optimal body growth during late gestation.

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STATEMENT OF COMPETING FINANCIAL INTERESTS

None

REFERENCES

ALMEIDA, J. R. & MANDARIM-DE-LACERDA, C. A. 2005. Maternal gestational protein-calorie restriction decreases the number of glomeruli and causes glomerular hypertrophy in adult hypertensive rats. *Am J Obstet Gynecol,* 192, 945-51.

AWAD, H., EL-SAFTY, I., EL-BARBARY, M. & IMAM, S. 2002. Evaluation of renal glomerular and tubular functional and structural integrity in neonates. *Am J Med Sci,* 324, 261-6.

BARKER, D. J. 1995. Intrauterine programming of adult disease. *Mol Med Today,* 1, 418-23.

BASSAN, H., TREJO, L. L., KARIV, N., BASSAN, M., BERGER, E., FATTAL, A., GOZES, I. & HAREL, S. 2000. Experimental intrauterine growth retardation alters renal development. *Pediatric Nephrology,* 15, 192-195.

BEHRMAN, R. E., LEES, M. H., PETERSON, E. N., DE LANNOY, C. W. & SEEDS, A. E. 1970. Distribution of the circulation in the normal and asphyxiated fetal primate. *Am J Obstet Gynecol,* 108, 956-69.

EMAMIAN, S. A., NIELSEN, M. B., PEDERSEN, J. F. & YTTE, L. 1993. Kidney dimensions at sonography: correlation with age, sex, and habitus in 665 adult volunteers. *AJR Am J Roentgenol,* 160, 83-6.

FELL, J. M., THAKKAR, H., NEWMAN, D. J. & PRICE, C. P. 1997. Measurement of albumin and low molecular weight proteins in the urine of newborn infants using a cotton wool ball collection method. *Acta Paediatr,* 86, 518-22.

FONG, D., DENTON, K. M., MORITZ, K. M., EVANS, R. & SINGH, R. R. 2014. Compensatory responses to nephron deficiency: adaptive or maladaptive? *Nephrology (Carlton),* 19, 119-28.

GALLINI, F., MAGGIO, L., ROMAGNOLI, C., MARROCCO, G. & TORTOROLO, G. 2000. Progression of renal function in preterm neonates with gestational age < or = 32 weeks. *Pediatr Nephrol,* 15, 119-24.

GUBHAJU, L., SUTHERLAND, M. R., HORNE, R. S., MEDHURST, A., KENT, A. L., RAMSDEN, A., MOORE, L., SINGH, G., HOY, W. E. & BLACK, M. J. 2014. Assessment of renal functional maturation and injury in preterm neonates during the first month of life. *Am J Physiol Renal Physiol,* 307, F149-58.

HINCHLIFFE, S. A., LYNCH, M. R., SARGENT, P. H., HOWARD, C. V. & VAN VELZEN, D. 1992. The effect of intrauterine growth retardation on the development of renal nephrons. *Br J Obstet Gynaecol,* 99, 296-301.

HINCHLIFFE, S. A., SARGENT, P. H., HOWARD, C. V., CHAN, Y. F. & VAN VELZEN, D. 1991. Human intrauterine renal growth expressed in absolute number of glomeruli assessed by the disector method and Cavalieri principle. *Lab Invest,* 64, 777-84.

HUGHSON, M., FARRIS, A. B., 3RD, DOUGLAS-DENTON, R., HOY, W. E. & BERTRAM, J. F. 2003. Glomerular number and size in autopsy kidneys: the relationship to birth weight. *Kidney Int,* 63, 2113-22.

KIM, J. H., KIM, M. J., LIM, S. H., KIM, J. & LEE, M. J. 2013. Length and volume of morphologically normal kidneys in korean children: ultrasound measurement and estimation using body size. *Korean J Radiol,* 14, 677-82.

KONJE, J. C., BELL, S. C., MORTON, J. J., DE CHAZAL, R. & TAYLOR, D. J. 1996. Human fetal kidney morphometry during gestation and the relationship between weight, kidney morphometry and plasma active renin concentration at birth. *Clin Sci,* 91, 169-75.

LUCAS, S. R., COSTA SILVA, V. L., MIRAGLIA, S. M. & ZALADEK GIL, F. 1997. Functional and morphometric evaluation of offspring kidney after intrauterine undernutrition. *Pediatr Nephrol,* 11, 719-23.

MANALICH, R., REYES, L., HERRERA, M., MELENDI, C. & FUNDORA, I. 2000. Relationship between weight at birth and the number and size of renal glomeruli in humans: a histomorphometric study. *Kidney Int,* 58, 770-3.

MORITZ, K. M., SINGH, R. R., PROBYN, M. E. & DENTON, K. M. 2009. Developmental programming of a reduced nephron endowment: more than just a baby's birth weight. *Am J Physiol Renal Physiol,* 296, F1-9.

NYENGAARD, J. R. & MARCUSSEN, N. 1993. The number of glomerular capillaries estimated by an unbiased and efficient stereological method. *J Microsc.,* 171, 27-37.

OSATHANONDH, V. & POTTER, E. 1963. Development of human kidney as shown by microdissection. III. Formation and interrelationship of collecting tubules and nephrons. *Archives of Pathology,* 76, 290-302.

RODRIGUEZ-SORIANO, J., AGUIRRE, M., OLIVEROS, R. & VALLO, A. 2005. Long-term renal follow-up of extremely low birth weight infants. *Pediatr Nephrol,* 20, 579-84.

RODRIGUEZ, M. M., GOMEZ, A. H., ABITBOL, C. L., CHANDAR, J. J., DUARA, S. & ZILLERUELO, G. E. 2004. Histomorphometric analysis of postnatal glomerulogenesis in extremely preterm infants. *Pediatr Dev Pathol,* 7, 17-25.

SATYAPAL, K. S., KALIDEEN, J. M., SINGH, B., HAFFEJEE, A. A. & ROBBS, J. V. 2003. Why we use the donor left kidney in live related transplantation. *S Afr J Surg,* 41, 24-6.

SCHREUDER, M., DELEMARRE-VAN DE WAAL, H. & VAN WIJK, A. 2006. Consequences of Intrauterine Growth Restriction for the Kidney. *Kidney and Blood Pressure Research,* 29, 108-125.

SILVER, L. E., DECAMPS, P. J., KORST, L. M., PLATT, L. D. & CASTRO, L. C. 2003. Intrauterine growth restriction is accompanied by decreased renal volume in the human fetus. *American Journal of Obstetrics and Gynecology,* 188, 1320-1325.

SUTHERLAND, M. R., GUBHAJU, L., MOORE, L., KENT, A. L., DAHLSTROM, J. E., HORNE, R. S., HOY, W. E., BERTRAM, J. F. & BLACK, M. J. 2011. Accelerated maturation and abnormal morphology in the preterm neonatal kidney. *J Am Soc Nephrol,* 22, 1365-74.

THAYYIL, S., SHEIK, S., KEMPLEY, S. T. & SINHA, A. 2008. A gestation- and postnatal age-based reference chart for assessing renal function in extremely premature infants. *J Perinatol,* 28, 226-9.

WANG, Y. P., CHEN, X., ZHANG, Z. K., CUI, H. Y., WANG, P. & WANG, Y. 2015. Effects of a restricted fetal growth environment on human kidney morphology, cell apoptosis and gene expression. *J Renin Angiotensin Aldosterone Syst,* 16, 1028-35.

WEITZ, M., LICHT, C., MULLER, M. & HABER, P. 2013. Renal ultrasound volume in children with primary vesicoureteral reflux allows functional assessment. *J Pediatr Urol,* 9, 1077-83.

WHITE, S. L., PERKOVIC, V., CASS, A., CHANG, C. L., POULTER, N. R., SPECTOR, T., HAYSOM, L., CRAIG, J. C., SALMI, I. A., CHADBAN, S. J. & HUXLEY, R. R. 2009. Is low birth weight an antecedent of CKD in later life? A systematic review of observational studies. *Am J Kidney Dis,* 54, 248-61.

WOODS, L., WEEKS, D. & RASCH, R. 2004. Programming of adult blood pressure by maternal protein restriction: role of nephrogenesis. *Kidney International,* 65, 1339-48.

ZOHDI, V., MORITZ, K. M., BUBB, K. J., COCK, M. L., WREFORD, N., HARDING, R. & BLACK, M. J. 2007. Nephrogenesis and the renal renin-angiotensin system in fetal sheep: effects of intrauterine growth restriction during late gestation. *Am J Physiol Regul Integr Comp Physiol,* 293, R1267-73.

ZOHDI, V., SUTHERLAND, M. R., LIM, K., GUBHAJU, L., ZIMANYI, M. A. & BLACK, M. J. 2012. Low Birth Weight due to Intrauterine Growth Restriction and/or Preterm Birth: Effects on Nephron Number and Long-Term Renal Health. *Int J Nephrol,* 2012, 136942.

FIGURES AND TABLES

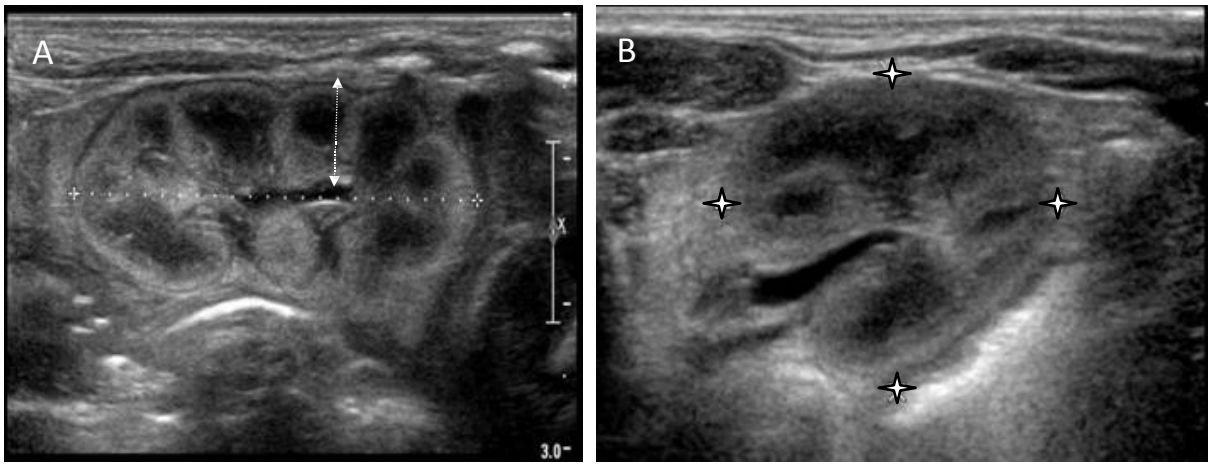

Figure 1: Ultrasound images of the preterm kidney. Kidney length (A), height and width (B) and cortical depth (overlay arrow- A) was measured with high resolution linear ultrasound.

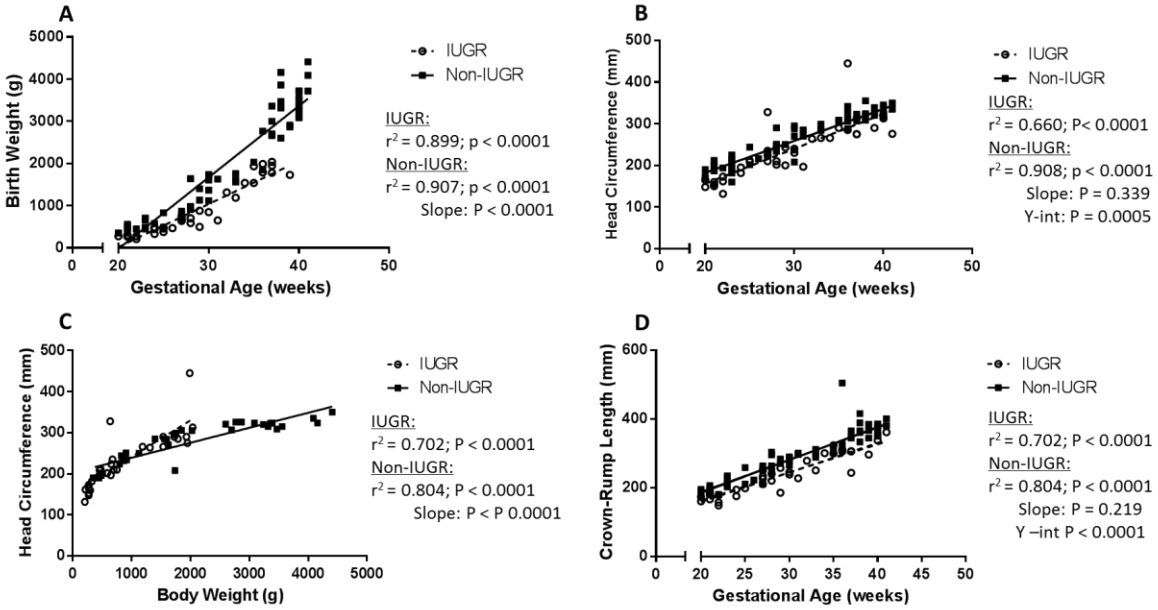

Figure 2: Body weights. Linear regression analyses of birth weight *versus* gestational age (A), head circumference *versus* gestational age (B), head circumference *versus* body weight (C) and crown-rump length *versus* gestational age (D) in IUGR (o) and non-IUGR (■) infants from 20 to 41 weeks' gestation. Regression lines for IUGR infants are represented as dashed lines and regression lines for the non-IUGR infants are represented as solid lines.

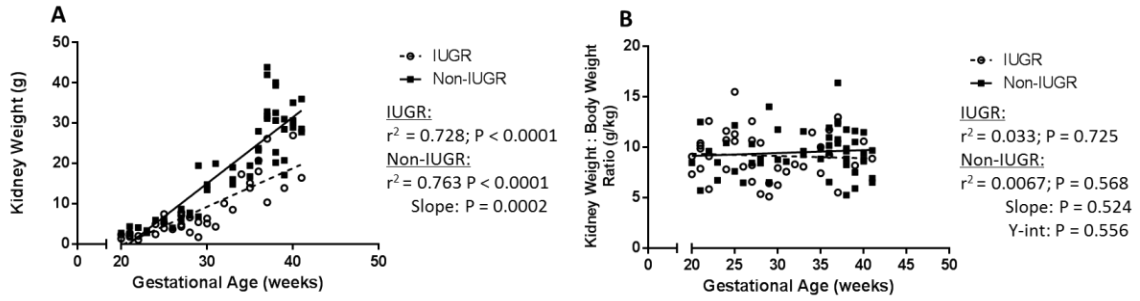

Figure 3: Kidney weights. Linear regression analyses of kidney weight versus gestational age (A) and kidney weight *versus* body weight (B) in IUGR (o) and non-IUGR (■) infants from 20 to 41 weeks of gestation. Regression lines for IUGR infants are represented as dashed lines and regression lines for the non-IUGR infants are represented as solid lines.

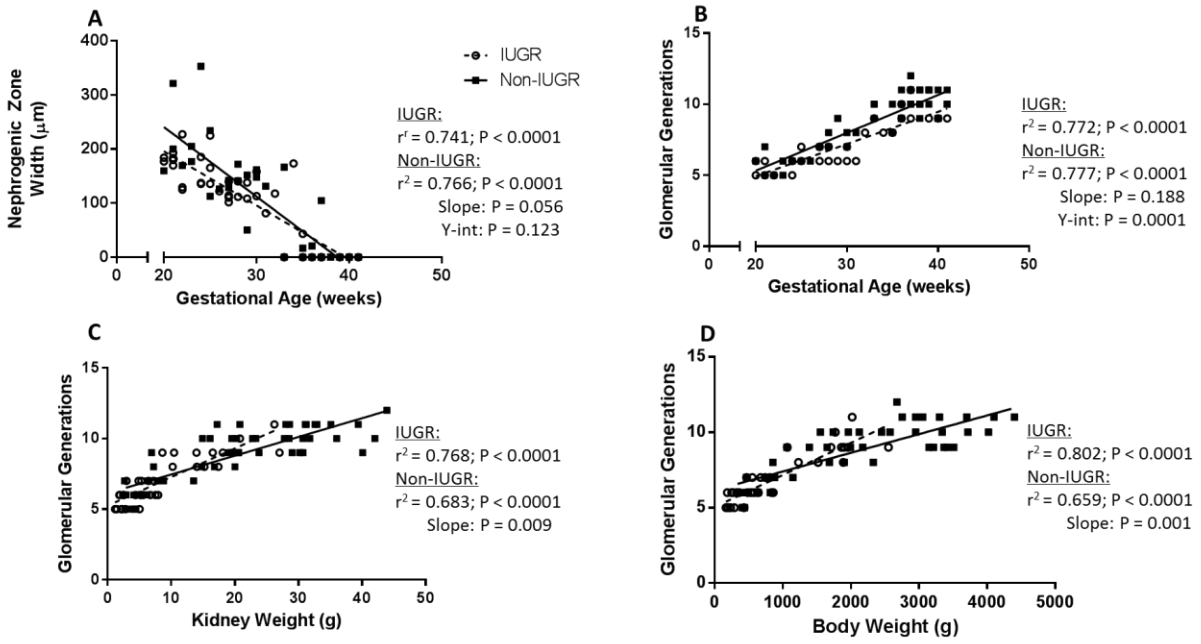

Figure 4: Nephrogenic zone width and number of glomerular generations formed in the developing kidneys. Linear regression analyses of nephrogenic zone width *versus* gestational age (A) in IUGR (o) and non-IUGR (■) infants, and linear regression analyses of number of glomerular generations *versus* gestational age (B), kidney weight (C), and body weight (D) in IUGR (o) and non-IUGR (■) infants. Regression lines for IUGR infants are represented as dashed lines and regression lines for the non-IUGR infants are represented as solid lines.

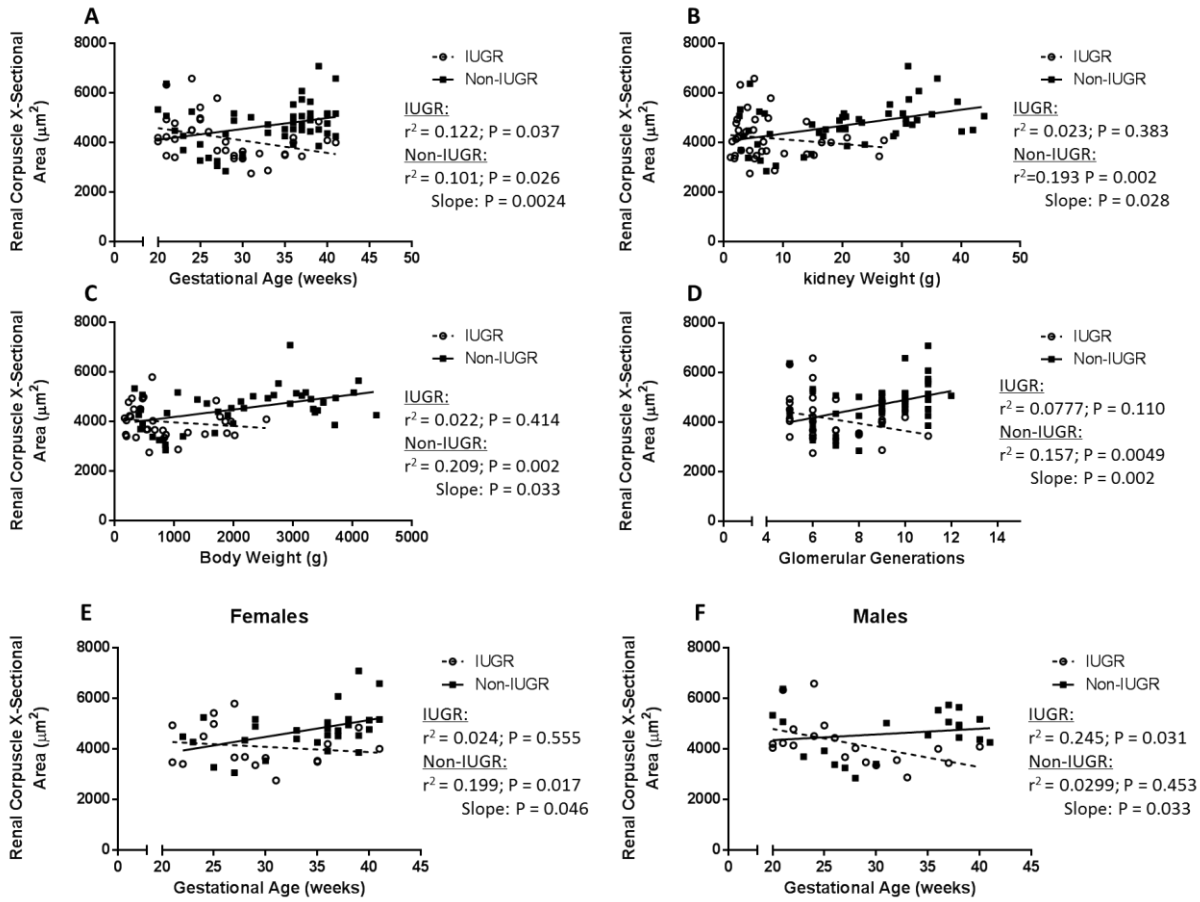

Figure 5: Renal corpuscle size. Linear regression analyses of renal corpuscle cross-sectional area *versus* gestational age (A), kidney weight (B), body weight (C) and number of glomerular generations (D) in IUGR (o) and non-IUGR (■). In panels E and F renal corpuscle cross-sectional area *versus* gestational age is shown specifically for the female (E) and male (F) IUGR and non-IUGR infants. Regression lines for IUGR infants are represented as dashed lines and regression lines for the non-IUGR infants are represented as solid lines.

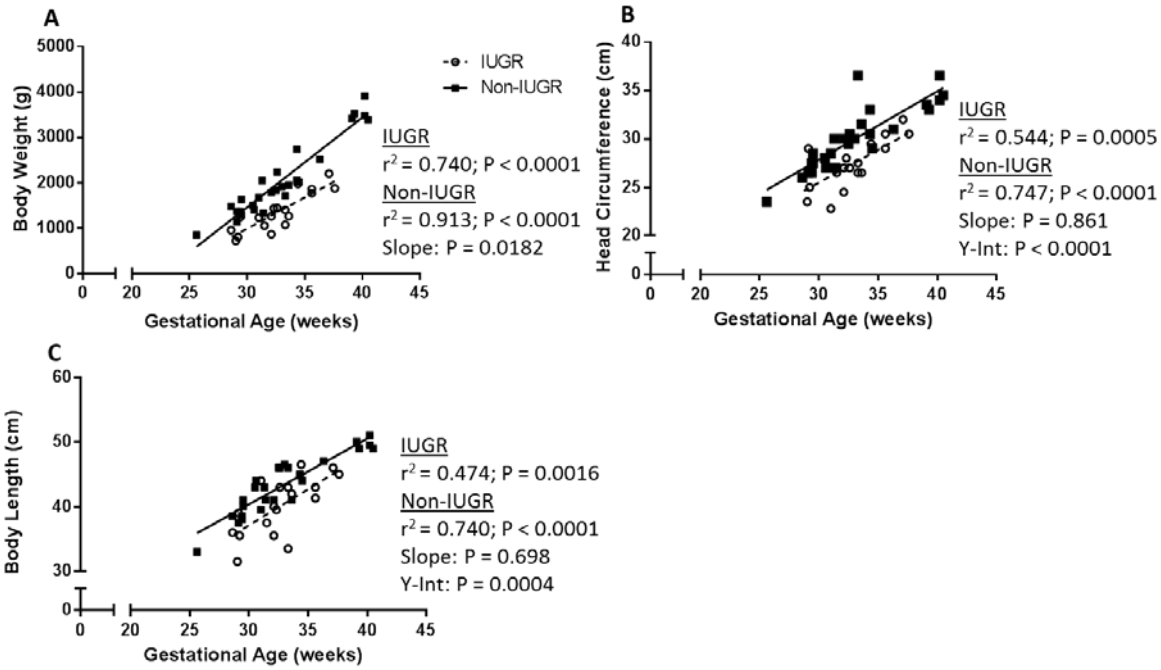

Figure 6. Linear regression analyses of body weight (A), head circumference (B), and body length (C) in IUGR (o) and non-IUGR (■) infants relative to gestational age at birth. Regression lines for IUGR infants are represented as dashed lines and regression lines for the non-IUGR infants are represented as solid lines.

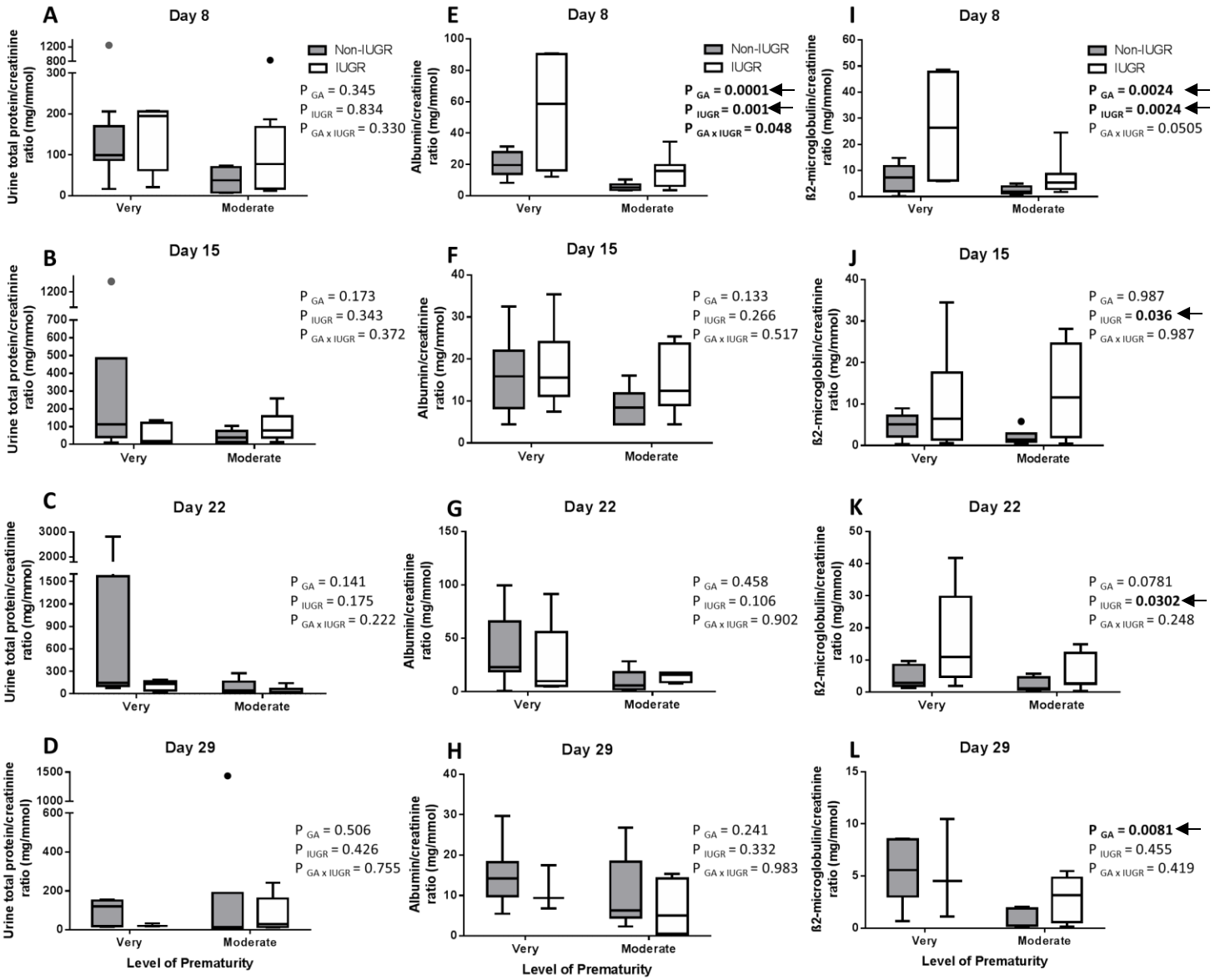
A
Day 8
Urine total protein/creatinine ratio (mg/mmol)
Non-IUGR
IUGR
P GA = 0.345
P IUGR = 0.834
P GA x IUGR = 0.330
Very
Moderate
E
Day 8
Albumin/creatinine ratio (mg/mmol)
P GA = 0.0001
P IUGR = 0.001
P GA x IUGR = 0.048
I
Day 8
ß2-microglobulin/creatinine ratio (mg/mmol)
P GA = 0.0024
P IUGR = 0.0024
P GA x IUGR = 0.0505
B
Day 15
P GA = 0.173
P IUGR = 0.343
P GA x IUGR = 0.372
F
Day 15
P GA = 0.133
P IUGR = 0.266
P GA x IUGR = 0.517
J
Day 15
ß2-microgloblin/creatinine ratio (mg/mmol)
P GA = 0.987
P IUGR = 0.036
P GA x IUGR = 0.987
C
Day 22
P GA = 0.141
P IUGR = 0.175
P GA x IUGR = 0.222
G
Day 22
P GA = 0.458
P IUGR = 0.106
P GA x IUGR = 0.902
K
Day 22
ß2-microglobulin/creatinine ratio (mg/mmol)
P GA = 0.0781
P IUGR = 0.0302
P GA x IUGR = 0.248
D
Day 29
P GA = 0.506
P IUGR = 0.426
P GA x IUGR = 0.755
Level of Prematurity
H
Day 29
P GA = 0.241
P IUGR = 0.332
P GA x IUGR = 0.983
Level of Prematurity
L
Day 29
P GA = 0.0081
P IUGR = 0.455
P GA x IUGR = 0.419
Level of Prematurity

Figure 7. Protein excretion over the first month of life. Urine total protein/creatinine (A-D), albumin/creatinine (E-H), and β2-microglobulin/creatinine (I-L) on postnatal days 8, 15, 22 and 29 in very and moderately preterm neonates. Box plots represents the interquartile ranges (IQR: 25^{th} and 75^{th} percentiles) and median (50%), with whiskers extending to 1.5 times the IQR from the box edges; outliers represented by (●). Data were analysed using a two-way ANOVA with the factors gestational age at birth ($P_{GA;}$ very preterm *versus* moderately preterm), intrauterine growth restriction ($P_{IUGR;}$ IUGR *versus* non-IUGR), and their interaction ($P_{GA \times IUGR}$).

Table 1: Timing of the cessation of nephrogenesis: Timing of the cessation of nephrogenesis in the kidneys of all IUGR infants and non-IUGR infants. Table shows whether nephrogenesis has ceased or was ongoing at the time of autopsy for all infants examined from 31 weeks of gestation or more. Nephrogenesis ongoing at the time of analysis is indicated by '✓'. Nephrogenesis complete at the time of analysis is indicated by 'X'

Gestational age (weeks)	Nephrogenesis ongoing	
	IUGR	Non-IUGR
20-30	Nephrogenesis ongoing in all infants (n= 26)	Nephrogenesis ongoing in all infants (n= 18)
31	✓	✓
32	✓	-
33	X	✓✓X
34	✓	-
35	✓X	✓X
36	XX	✓ X X X X
37	XX	✓ X X X X
38	-	X X X X X X
39	X	X X X X
40	X	X X X X
41	X	X X X

Table 2. Infant demographics. Birth weight, body length, head circumference, body-surface area (BSA), and proportion of males and twins in the very preterm (IUGR and non-IUGR) infants and moderately preterm (IUGR and non-IUGR) infants. Data relating to birth weight, body length, head circumference and BSA are presented as the median [Interquartile range] and mean ± SD.

* Denotes $p < 0.05$ IUGR *versus* non-IUGR grouping.

:

	Birth Weight (g)	Body Length (cm)	Head Circumference (cm)	BSA	Male (%)	Twins (%)
Very preterm (26-31 weeks gestation)						
Non-IUGR (n = 11) Median (min, max) Mean ± SD	1367 (847, 1675) 1375 ± 220.59	39.5 (33, 44) 39.45 ± 2.96	27 (23.5, 28.5) 26.95 ± 1.40	0.12 (0.08, 0.13) 0.118 ± 0.02	36	55
IUGR (n = 6) Median (min, max) Mean ± SD	*1005 (719, 1233) 984.67 ± 197.04	36.75 (31.5, 44) 37.25 ± 4.16	25.5 (22.8, 29) 25.47 ± 2.24	0.10 (0.08, 0.12) 0.102 ± 2.40	14	57
Moderately preterm (32-37 weeks gestation)						
Non-IUGR (n = 10) Median (min, max) Mean ± SD	1983 (1710, 2741) 2076.6 ± 331.46	45.5 (41, 47) 44.73 ± 2.15	30.5 (29, 36.5) 31.15 ± 2.19	0.15 (0.14, 0.17) 0.153 ± 0.011	80	20
IUGR (n = 10) Median (min, max) Mean ± SD	*1419 (863, 1978) 1435 ± 350.71	*41.65 (33.5, 46.5) 40.73 ± 3.83	*27.25 (24.5, 30.5) 27.6 ± 1.73	0.13 (0.09, 0.16) 0.124 ± 0.022	60	40

Table 3. Kidney growth over the first month of life. Kidney parameters including; volume, length, depth, width and cortical depth, in very preterm (IUGR and non-IUGR) groups and moderately preterm (IUGR and non-IUGR) infants over the first month of life. Values represent the mean ± SD. Data were assessed by a two-way ANOVA with the factors of gestational age at birth ($P_{GA;}$ very preterm *versus* moderately preterm), intrauterine growth restriction ($P_{IUGR;}$ IUGR *versus* non-IUGR), and their interaction ($P_{GA \times IUGR}$). *P < 0.05 IUGR *versus* non-IUGR within the gestational age grouping. †P < 0.05 very preterm *versus* moderately preterm within IUGR and non-IUGR group.

Kidney measurements	Very preterm non-IUGR	Very preterm IUGR	Moderately preterm non-IUGR	Moderately preterm IUGR	Two–way ANOVA P - values
DAY 8					
Right Kidney					
Kidney length (cm)	†3.36 ± 0.33 (n = 7)	3.49 ± 0.38 (n = 6)	†4.05 ± 0.14 (n = 8)	3.80 ± 0.52 (n = 6)	$P_{GA} = 0.0028$ ← $P_{IUGR} = 0.9323$ $P_{GA \times IUGR} = 0.4036$
Kidney width (cm)	†2.13 ± 0.25 (n = 7)	1.90 ± 0.16 (n = 6)	†2.46 ± 0.42 (n = 8)	2.20 ± 0.34 (n = 6)	$P_{GA} = 0.0036$ ← $P_{IUGR} = 0.0716$ $P_{GA \times IUGR} = 0.9252$
Kidney depth (cm)	*1.89 ± 0.27 (n = 7)	*1.54 ± 0.20 (n = 6)	1.95 ± 3.72 (n = 8)	1.83 ± 0.44 (n = 6)	$P_{GA} = 0.1420$ $P_{IUGR} = 0.1755$ $P_{GA \times IUGR} = 0.2769$
Kidney volume (cm^3)	7.19 ± 1.83 (n = 7)	5.45 ± 1.36 (n = 6)	10.44 ± 0.41 (n = 8)	8.46 ± 3.71 (n = 6)	$P_{GA} = 0.0068$ ← $P_{IUGR} = 0.2450$ $P_{GA \times IUGR} = 0.7943$
Kidney cortical depth (cm)	†0.63 ± 0.13 (n = 7)	0.68 ± 0.15 (n = 6)	†0.80 ± 0.27 (n = 7)	0.78 ± 0.21 (n = 6)	$P_{GA} = 0.0308$ ← $P_{IUGR} = 0.6719$ $P_{GA \times IUGR} = 0.6444$
Left Kidney					
Kidney length (cm)	†3.47 ± 0.40 (n = 7)	3.56 ± 0.32 (n = 6)	†4.19 ± 0.39 (n = 8)	3.85 ± 0.38 (n = 6)	$P_{GA} = 0.0015$ ← $P_{IUGR} = 0.6773$ $P_{GA \times IUGR} = 0.3376$
Kidney width (cm)	2.21 ± 0.46 (n = 7)	†1.93 ± 0.14 (n = 6)	2.30 ± 0.39 (n = 8)	†2.39 ± 0.41 (n = 6)	$P_{GA} = 0.0709$ $P_{IUGR} = 0.5134$ $P_{GA \times IUGR} = 0.1945$
Kidney depth (cm)	*1.74 ± 0.21 (n = 7)	*†1.33 ± 0.27 (n = 6)	1.8 ± 0.31 (n = 8)	†1.76 ± 0.36 (n = 6)	$P_{GA} = 0.0228$ ← $P_{IUGR} = 0.1290$ $P_{GA \times IUGR} = 0.0715$
Kidney volume (cm^3)	7.26 ± 2.62 (n = 7)	4.89 ± 1.60 (n = 6)	9.36 ± 3.66 (n = 8)	8.87 ± 3.31 (n = 6)	$P_{GA} = 0.0564$ ← $P_{IUGR} = 0.1978$ $P_{GA \times IUGR} = 0.6635$
Kidney cortical depth (cm)	0.64 ± 0.11 (n = 7)	0.68 ± 0.15 (n = 6)	0.72 ± 0.16 (n = 7)	0.72 ± 0.11 (n = 6)	$P_{GA} = 0.2600$ $P_{IUGR} = 0.7038$ $P_{GA \times IUGR} = 0.7915$
DAY 29					
Right Kidney					
Kidney length (cm)	3.85 ± 0.38 (n = 7)	3.64 ± 0.54 (n = 4)	4.31 ± 0.31 (n = 4)	4.04 ± 0.38 (n = 7)	$P_{GA} = 0.0232$ ← $P_{IUGR} = 0.1964$ $P_{GA \times IUGR} = 0.8589$
Kidney width (cm)	*2.26 ± 0.22 (n = 7)	*1.96 ± 0.21 (n = 4)	2.60 ± 0.46 (n = 4)	2.24 ± 0.25 (n = 7)	$P_{GA} = 0.0190$ ← $p_{IUGR} = 0.0142$ ← $P_{GA \times IUGR} = 0.8191$
Kidney depth (cm)	1.90 ± 0.26 (n = 7)	1.67 ± 0.24 (n = 4)	2.15 ± 0.29 (n = 4)	1.97 ± 0.37 (n = 7)	$P_{GA} = 0.0526$ ← $P_{IUGR} = 0.1323$ $P_{GA \times IUGR} = 0.8514$

Kidney volume (cm^3)	8.77 ± 2.14 (n = 7)	6.35 ± 2.02 (n = 4)	12.78 ± 4.01 (n = 4)	9.56 ± 3.10 (n = 7)	$P_{GA} = 0.0086$ ← $p_{IUGR} = 0.0336$ ← $P_{GA \times IUGR} = 0.7489$
Kidney cortical depth (cm)	0.90 ± 0.17 (n = 7)	0.78 ± 0.08 (n = 4)	0.92 ± 0.11 (n = 4)	0.80 ± 0.22 (n = 7)	$P_{GA} = 0.8296$ $P_{IUGR} = 0.1159$ $P_{GA \times IUGR} = 0.9705$
Left Kidney					
Kidney length (cm)	3.64 ± 0.38 (n = 7)	3.62 ± 0.26 (n = 4)	4.41 ± 0.29 (n = 4)	4.02 ± 0.30 (n = 7)	$P_{GA} = 0.0009$ ← $P_{IUGR} = 0.2020$ $P_{GA \times IUGR} = 0.2211$
Kidney width (cm)	2.42 ± 0.31 (n = 7)	2.17 ± 0.23 (n = 4)	2.46 ± 0.33 (n = 4)	2.37 ± 0.24 (n = 7)	$P_{GA} = 0.3624$ $P_{IUGR} = 0.2063$ $P_{GA \times IUGR} = 0.5226$
Kidney depth (cm)	1.81 ± 0.29 (n = 7)	1.61 ± 0.31 (n = 4)	1.90 ± 0.29 (n = 4)	1.77 ± 0.27 (n = 7)	$P_{GA} = 0.3811$ $P_{IUGR} = 0.1930$ $P_{GA \times IUGR} = 0.7372$
Kidney volume (cm^3)	8.41 ± 2.00 (n = 7)	6.64 ± 1.83 (n = 4)	10.86 ± 2.78 (n = 4)	9.02 ± 2.61 (n = 7)	$P_{GA} = 0.0279$ ← $P_{IUGR} = 0.0912$ $P_{GA \times IUGR} = 0.9719$
Kidney cortical depth (cm)	0.79 ± 0.12 (n = 7)	0.79 ± 0.05 (n = 4)	0.93 ± 0.07 (n = 4)	0.85 ± 0.26 (n = 7)	$P_{GA} = 0.1916$ $P_{IUGR} = 0.5670$ $P_{GA \times IUGR} = 0.6417$

Chapter Six:

RENAL IMPAIRMENT ALREADY EVIDENT WITHIN THE FIRST MONTH OF LIFE IN PRETERM INDIGENOUS AUSTRALIANS

CHAPTER SIX DECLARATION

Declaration by candidate

[It is to be noted that I have used my married surname (Ryan) in this manuscript]

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

Nature of contribution	Extent of contribution (%)
I conducted all of the experimental work and data analyses. I also wrote the manuscript.	80%

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

Name	Nature of contribution	Extent of contribution (%) for student co-authors only	Co-author(s), Monash student Y/N
Megan Sutherland, Belinda Davison, Shanti Diwarkarla, Wendy Hoy, Gurmeet Singh, M. Jane Black	Involved in the design of experiments, obtained funding, involved in infant recruitment and urine collection, assisted in editing the manuscript		N

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

Candidate's Signature	[redacted]	Date 29.9.16
Main Supervisor's Signature	[redacted]	Date 29.9.16

RENAL IMPAIRMENT ALREADY EVIDENT WITHIN THE FIRST MONTH OF LIFE IN PRETERM INDIGENOUS AUSTRALIANS

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ABSTRACT

There is currently an epidemic of renal disease in Indigenous Australians with the age-adjusted rate of end stage kidney disease (ESKD) ~10 times greater in Indigenous Australians than in non-Indigenous Australians when considering deaths and hospitalisations. The cause of the high incidence of ESKD amongst the Indigenous population is multifactorial, however, recent evidence suggests that the antecedents may originate very early in life. Therefore, in this study, we compared the levels of renal injury in the early postnatal period after birth (at postnatal day 4) and after a month of life (postnatal day 29) in 60 Indigenous and 43 non-Indigenous Australian preterm infants. Spot urine samples were collected on days 8, 15, 22 and 29 after birth, and were used to measure levels of urinary total protein (g/L), albumin (mg/L) and β2-microglobulin (mg/L), which were all corrected for urine creatinine (μmol/L) levels. The findings of this study support the concept that the origins of the high incidence of renal disease in Indigenous Australians in adulthood may originate very early in life. Indigenous preterm infants' were more prevalent to abnormally high levels of cystatin-C and neutrophil gelatinase - associated ligase (NGAL), at 4 days of age. Pathological proteinuria was evident in 13% of Indigenous infants in the first month of life, compared to 4% of non-Indigenous infants. In addition, the majority of infants exhibiting 2 or more abnormal urine measurements over the first month of life (and thus considered at 'high risk' of developing renal dysfunction) were identified as Indigenous. Overall, the findings of this study, demonstrate that the early neonatal renal dysfunction is exacerbated in Indigenous infants born preterm and there is evidence of greater renal injury.

INTRODUCTION

There is currently an epidemic of renal disease in Indigenous Australians (Aboriginal and Torres Strait Islanders), with chronic kidney disease being 5 times more prevalent compared with non-Indigenous Australians (AIHW, 2014). In the most recent ANZDATA 2013-2014 data, the age-adjusted rate of end stage kidney disease (ESKD) was ~10 times greater in Indigenous Australians than in non-Indigenous Australians when considering deaths and hospitalisations (ANZDATA, 2015). The cause of the high incidence of ESKD amongst the Indigenous population is multifactorial (AIHW, 2015), however, recent evidence suggests that the antecedents may originate very early in life (Spencer et al., 2001, Hoy et al., 2006). Indeed, it is suggested that the high incidence of renal disease in the Indigenous population may be a legacy of the improved neonatal survival over recent decades of infants that were born of low birth weight (Hoy et al., 2006). Although a recent success story in neonatal medicine, the marked improvement in the survival of extremely and very low birth weight infants is linked to long-term deleterious renal consequences (White et al., 2009). Indeed, there have been a multitude of epidemiological studies linking low birth weight with adult renal disease and a meta-analysis of 31 of these studies reported a 70% increased risk of developing chronic renal disease in subjects born of low birth weight (White et al., 2009).

Low birth weight can result from preterm birth (birth prior to 37 completed weeks of gestation) and/or intrauterine growth restriction (IUGR). In the Indigenous population, there is both a high incidence of IUGR (approximately 12% of births compared with ~8% in the non-Indigenous population), and preterm birth (approximately 13.5% of births compared with ~6% in the non-Indigenous population) (Clarke and Boyle, 2014).

Due to their relative renal immaturity, preterm infants experience a low glomerular filtration rate (GFR) and thus, low creatinine clearance. Creatinine clearance increases with gestational

age at birth and with postnatal age (Bueva and Guignard, 1994, Gubhaju et al., 2014). In contrast, the fractional excretion of sodium is inversely correlated with gestational age at birth and postnatal age (Bueva and Guignard, 1994, Gallini et al., 2000, Gubhaju et al., 2014). The kidneys of preterm newborns are particularly vulnerable to injury in the neonatal period because of the increased functional demands and increased renal blood flow that occurs postnatally, as well as exposure to nephrotoxic medications used in their neonatal care (Nagai and Takano, 2004, Gilbert et al., 1996). As expected, the levels of renal injury increase with the severity of prematurity; for instance, the urinary levels of both albumin and β2-microglobulin (a marker of tubular injury) increase with decreasing gestational age at birth (Tsukahara et al., 1990, Tsukahara et al., 1994, Gubhaju et al., 2014).

In this study, we compared the levels of renal injury in the early postnatal period after birth (at postnatal day 4) and after a month of life (postnatal day 29) in Indigenous and non-Indigenous Australian preterm and term infants. The presence of early renal injury was assessed using the biomarkers urinary cystatin-C and urinary NGAL, measured at day 4 of postnatal life. In addition, the concentration of protein excretion in the urine was measured to determine the presence of glomerular injury (elevated albumin) and/or tubular injury (elevated β2-microglobulin) on postnatal days 8, 15, 22, and 29 of life. The preterm infants were stratified according to gestational age at birth: moderately preterm (32 - 36 weeks' gestation); very preterm (29 - 31 weeks' gestation), and extremely preterm ($\leq$ 28 weeks' gestation). Given the high prevalence of renal disease in adult Indigenous Australians, we hypothesised that Indigenous infants would be more vulnerable to renal impairment following preterm birth. The findings have important clinical implications, given that the neonatal period provides a critical window for intervention as nephrogenesis is still ongoing in the majority of preterm infants during this period.

MATERIALS AND METHODS

Ethics Statement

Ethics approval to conduct this study was obtained from the Human Research Ethics Committee of the Northern Territory Department of Health Research (HREC). Written informed parental consent was obtained for all participants in the study.

Study Population

Preterm neonates (< 37 completed weeks of gestation) and term infants (born 37 - 41 weeks' gestation) were recruited into the study at the Royal Darwin Hospital, Northern Territory, Australia, during the period 2011 to 2015.

Over the study period, 119 neonates were recruited into the study and were grouped according to gestational age at birth: extremely preterm (≤ 28 weeks of gestation; Indigenous n = 17/119 [14.3%]; non-Indigenous n = 11/119 [9.24%]); very preterm (29-31 weeks of gestation; Indigenous n = 20/119 [16.8%]); non-Indigenous n = 10/119 [8.4%]); moderately preterm (32-36 weeks of gestation; Indigenous n = 23/119[19.3%]); non-Indigenous n = 22/119 [18.5%]) and term (n = 16/119 [13.45%]). Indigenous infants were identified as babies born to a mother who was recorded as a 'self-identified' Aboriginal Australian as noted in the Aboriginal Delivery Suite Register. Within the Indigenous cohorts, few mothers knew the date of their last menstrual period or had undertaken an early fetal ultrasound dating in the first trimester. Hence, the Dubowitz scoring system (Dubowitz et al., 1970), was used to determine the gestational age at delivery, by the neonatal paediatrician, within the first four days of postnatal life. Infants were not recruited into the study if there was evidence of congenital abnormalities.

A number of term infants were also recruited into the study (n = 16). The number of term Indigenous infants was low, as the majority of term Indigenous term babies are born within their communities. The data from the term infants (Indigenous and non-Indigenous) infants were grouped and used for comparison with data from the preterm infants.

Infant Recruitment and Study Design

Mothers were approached 48 hours after delivery to request the inclusion of their infant into the study. If maternal consent was given (93% of mothers that were approached consented), urine collections were undertaken at days 4, 8, 15, 22, and 29 after birth. At day 4, urinary cystatin-C and NGAL (measures of renal injury) were measured in urine extracted from infant diapers (using a method previously described (Heckmann et al., 2005)). Urinary cystatin-C and NGAL levels were measured in duplicates using a sandwich ELISA in microwells coated with a monoclonal antibody against human cystatin-C (cystatin-C ELISA kit, BioPoirto Diagnostics; Gentofte, Denmark); and a sandwich ELISA in microwells coated with a monoclonal antibody against human NGAL (NGAL ELISA kit, BioPoirto Diagnostics; Gentofte, Denmark) (Gubhaju et al., 2014).

Where practical, urinary protein, albumin, ß2-microglobulin, and creatinine were measured in urine spot samples at days 8, 15, 22, and 29. In some babies, it was not feasible to collect spot urine samples; for those infants, a cotton ball was placed within the diaper and proteins subsequently extracted using an established protocol (Fell et al., 1997). Urine analyses were performed by the Southern Health Pathology Department (Southern Cross Pathology; Clayton, Australia). Urine creatinine was measured with a modified Jaffe reaction colorimetry method, using a Beckman Coulter SYNCHRON LX20PRO® system, with reagents and calibrators supplied by Beckman Diagnostics (Sydney, Australia). Urine total protein (UTP), albumin, and β2-microglobulin were measured using nephelometric technology on a Beckman Coulter

immunochemistry system, with reagents and calibrators supplied by Beckman Diagnostics (UTP and albumin; Beckman Diagnostics; Sydney, Australia) and DakoCytomation (β2-microglobulin; DakoCytomation; Glostrup, Denmark) (Gubhaju et al., 2014). Urinary albumin / creatinine was used as an indicator of glomerular function and ß2-microglobulin / creatinine as an indicator of renal tubular function. In some babies, it was not possible to collect urine at every time point; this accounts for the variability in the number of data points at the different postnatal time points.

Statistical Analysis

Statistical analyses were performed using GrapPad Prism v6.04 for Windows and Intercooled Stata v14 for Windows. Data are presented as medians [IQR]. Statistical significance was accepted at the level of $P < 0.05$.

Birth characteristics (birth weight, body length and head circumference) and renal function data (urinary total protein, albumin, and β2-microglobulin-to-creatinine ratios, and urinary cystatin-C and NGAL levels) were analysed using a two-way analysis of variance (ANOVA), followed by a Bonferroni post hoc test. The factors assessed in all of these analyses were: degree of prematurity (P_{GA}), Indigenous ethnicity (P_{IND}), and their interaction ($P_{GA \times IND}$).

In the preterm infants, cystatin-C and NGAL levels were considered grossly abnormal if the values were greater than 1.5 times above the 75% confidence interval (Tukey, 1977) of those reported for the term infants. Grossly abnormal levels of urine total protein, albumin and β2-microglobulin in the preterm infants were defined as values greater than 1.5 times the 75% confidence interval within the gestational age group.

RESULTS

Birth characteristics of Indigenous and non-Indigenous infants

Birth characteristics of the preterm Indigenous and non-Indigenous infants are described in Table 1. Overall, there were a high proportion of male Indigenous infants in all the preterm categories (extremely, very, and moderately preterm). There were no differences in birth weight, body length, and head circumference relative to gestational age at birth between the Indigenous and non-Indigenous infants (Figure 1A-C). Notably, the Indigenous infants in the moderately preterm group weighed significantly less (P = 0.021) with a reduced head circumference (P = 0.048) compared with non-Indigenous infants; this was in accordance with the greater number of IUGR infants within the Indigenous moderately preterm group. In the very preterm category, although there were a higher proportion of IUGR infants in the Indigenous cohort, the median birth weights were similar, and there was no significant difference in the average birth weights between the Indigenous and non-Indigenous groups.

Assessing renal injury markers for early detection of kidney injury

Cystatin-C: At day 4 of life there was a significant decrease in urinary cystatin-C levels with increasing gestational age at birth (P < 0.0001) (Figure 2A); but there was no effect of Indigenous ethnicity. There was a wide range in cystatin-C levels in preterm infants ranging from <1 ng/ml to 240 ng/ml at day 4 of life. Abnormally high cystatin-C levels in the preterm infants were defined as 1.5 times above the 75th percentile (Tukey, 1977) for term infants ($\geq$ 39.5 ng/ml). A high proportion of the extremely and very preterm infants exhibited abnormally high cystatin-C levels at postnatal day 4, when compared with term infants (Figure 2A). Overall, the prevalence of abnormally high cystatin-C levels was greater in the Indigenous preterm infants (extremely preterm: Indigenous: n = 10/14 [71.1%]; non-Indigenous: n = 6/10 [60%]); very preterm infants: Indigenous: n = 12/21 [57.1%]; non-Indigenous n = 4/10 [40%], and moderately preterm infants: Indigenous: n = 8/23 [35%]; non-Indigenous: n = 3/22 [14%]).

NGAL: At day 4 of life there was a significant decrease in urinary NGAL levels with increasing gestational age at birth (P = 0.014) (Figure 2B). Overall, there was no effect of Indigenous ethnicity on urinary NGAL levels. There was a wide variation in NGAL levels in the preterm infants studied, ranging from <1 ng/ml to 495 ng/ml. Abnormally high levels of NGAL in the preterm infants were defined as being 1.5 times above the 75th percentile for the term infants ($\geq$ 27.3 ng/ml). Abnormal urinary NGAL levels were detected in 16 of 25 extremely preterm infants (Indigenous: n = 10/25 [40%]; non-Indigenous: n = 6/25 [24%]); 15 of 31 very preterm infants (indigenous: n = 14/31 [45.2%]; non-Indigenous: n = 1/31 [3.2%]) and 14 of 45 moderately preterm infants (Indigenous; n = 7/45 [15.6%]; non-Indigenous; n = 7/45 [15.6%]).

Assessment of renal function over the first month of life

Figure 3 shows the levels of urinary total protein/creatinine, albumin/creatinine and β2-microglobulin/creatinine in Indigenous and non-Indigenous infants born extremely, very, and moderately preterm at days 8, 15, 22, and 29 of life.

Overall, there were significant reductions in urinary total protein/creatinine (Figure 3 A-D), albumin/creatinine (Figure 3 E-H), and β2-microglobulin/creatinine (Figure 3 I- L) with increasing gestational age at birth. At the end of the first week of life (postnatal day 8), Indigenous infants demonstrated significant increases in albumin/creatinine and β2-microglobulin/creatinine levels (P = 0.006 and P = 0.003, respectively) compared with non-Indigenous infants, with increased β2-microglobulin/creatinine levels persisting at day 15; urine total protein/creatinine levels were also significantly greater in Indigenous compared with non-Indigenous infants at postnatal day 15.

Figure 4 shows the levels of urinary protein excretion with increasing postnatal age in Indigenous and non-Indigenous infants born extremely, very, and moderately preterm. There were no significant differences in protein excretion with increasing postnatal age within each

of the gestational age groups (Figure 4), with the exception of the extremely preterm infants where β2-microglobulin/creatinine levels significantly decreased over the first month of life (P = 0.0019; Figure 4C). Urinary total protein/creatinine levels were significantly higher in the Indigenous very preterm infants compared with the non-Indigenous infants, over the first month of life (P = 0.004; Figure 4A). In addition, in comparison with non-Indigenous infants, Indigenous extremely preterm and moderately preterm infants exhibited markedly higher levels of β2-microglobulin/creatinine over the first month of life (P = 0.0003 and P = 0.029, respectively)(Figure 4C). In non-Indigenous infants, β2-microglobulin/creatinine levels remained relatively low (~15mg/mmol) over the first month of life.

Pathological proteinuria: Over the 4 postnatal time-points studied, pathological proteinuria (urinary total protein $\geq$ 500 mg/l) was detected in 14 Indigenous (~13%) and 4 non-Indigenous (~4%) preterm infants.

Measurements of renal impairment: Seventeen preterm infants exhibited multiple measures ($\geq$ 2) of renal dysfunction at one or more postnatal time points, as presented in Table 2. Fourteen of these infants were Indigenous (23% of Indigenous preterm infants) and 3 were non-Indigenous (7% of non-Indigenous preterm infants). There were 10 infants considered as 'high risk' for renal impairment (defined as having >2 measures of renal injury). Of these, 8 were Indigenous infants (3 extremely preterm infants, 4 very preterm infants and 1 moderately preterm infant) and two were non-Indigenous extremely preterm infants.

DISCUSSION

The findings of this study clearly demonstrate the renal vulnerability of Indigenous Aboriginal Australian infants to preterm birth. It is well documented that there is renal dysfunction in preterm infants in the neonatal period following preterm birth, which is likely due to the increased postnatal functional demands in ill-prepared immature kidneys. Importantly,

however, the findings of this study, demonstrate that early neonatal renal dysfunction is exacerbated in Indigenous infants born preterm and there is evidence of greater renal injury. Hence, the greater vulnerability to renal impairment observed in the adult Indigenous Australian population is also evident very early in life in Indigenous infants born preterm.

Generally, renal impairment was much higher in the preterm Indigenous infants relative to the non-Indigenous infants, with a higher proportion of Indigenous preterm infants exhibiting abnormally high levels of cystatin-C (a marker of renal injury), at 4 days after birth. In addition, 13% of Indigenous infants exhibited pathological proteinuria in the first month of life, compared with 4% of non-Indigenous infants, indicative of severe renal dysfunction; pathological proteinuria was most evident in the Indigenous extremely and very preterm infants, followed by the extremely preterm non-Indigenous infants. Encouragingly, in all infants that exhibited proteinuria at the early time points, the abnormal readings were no longer apparent at the next time-point, indicative of renal repair. Of concern, however, the majority of infants exhibiting 2 or more abnormal urine measurements over the first month of life (and thus considered at 'high risk' of developing renal dysfunction) were identified as Indigenous. Notably, in our study, renal tubular function (as assessed by levels of ß2-microglobulin in the urine) appears to have been the most greatly affected by preterm birth, which is in accordance with the later timing of tubular maturation (which normally occurs late in gestation and extends into the postnatal period) (Fong et al., 2014). It is therefore not surprising that it was tubular function that was more greatly impacted upon by preterm birth. Notably, the levels of ß2-microglobulin excretion were significantly higher in the Indigenous preterm infants at postnatal days 8 and 15. In addition, abnormally high levels of cystatin-C at day 4 of life, were more prevalent in the Indigenous preterm infants; also indicative of early tubular dysfunction. There was also an increased prevalence of abnormally high urinary NGAL levels in Indigenous

preterm infants born extremely or very preterm at day 4 of life, but not observed in those born moderately preterm.

Indeed, urinary cystatin-C and NGAL have previously been validated as good markers of tubular dysfunction (Conti et al., 2006, Nauta et al., 2011). Cystatin-C is normally filtered, reabsorbed and metabolised completely by the proximal tubules with no evidence of tubular secretion. In the absence of tubular injury, NGAL is secreted in very small amounts by the renal proximal tubule cells, predominantly during early renal development (Kjeldsen et al., 1993). Therefore, cystatin-C and NGAL have been validated as good markers of tubular dysfunction and thus, increased levels of urinary cystatin-C and/or NGAL indicate impaired reabsorption within the renal tubules.

The findings of this study support the concept that the origins of the high incidence of renal disease in Indigenous Australians in adulthood may originate very early in life. Renal disease is one of the leading causes of morbidity in Indigenous Australians in adulthood, with rates of renal replacement therapy 8 to 9 times higher amongst the adult Indigenous populations than non-Indigenous (AIHW, 2011, ANZDATA, 2016), with even higher rates of more than 20- fold amongst the remote Indigenous communities (Cass et al., 2001, Preston-Thomas et al., 2007). As such, the treatments and subsequent clinical sequelae lead to a major financial burden on the Australian health system (You et al., 2002). Over recent decades, there has been mounting evidence to suggest that the escalating incidence of renal disease in Indigenous Australians may be the legacy of the improved survival of preterm and/or IUGR infants at the beginning of life (Sayers and Powers, 1993, Hoy and Nicol, 2010). Indeed, with the marked improvements in neonatal care there has been a dramatic improvement in the survival of preterm infants, with infants born as early as 22-25 weeks now having a 50% or greater chance of survival (Beck et al., 2010). It is proposed that the induction of renal injury and impairment of renal function

in the early postnatal period following IUGR and preterm birth is likely to predispose to renal disease later in life, with meta-analyses demonstrating clear links between low birth weight and adult renal disease (White et al., 2009). Our findings certainly support the concept of early life programming of renal disease, and furthermore provide a plausible explanation for the increased prevalence of renal disease in Indigenous Australians in adulthood, given that there is a much higher incidence of IUGR and preterm birth in the Indigenous population, coupled with their greater renal vulnerability when preterm birth occurs (as shown in this study).

Overall, there was very high recruitment of infants, with 93% of parents providing consent for their infants to be included in the study. Interestingly, over the study period, there was a high proportion of males born preterm in all the preterm categories (extremely, very, and moderately preterm) in the Indigenous population. These findings are in accordance with a number of studies that show that males are more likely to be preterm than females (Kent et al., 2012). Interestingly, the higher prevalence of males born preterm was not observed in the non-Indigenous preterm infants, with relatively similar numbers of males and females within each of the gestational age categories. There is some evidence to suggest that males are developmentally delayed compared with females and a 'male disadvantage' following preterm birth is well described (Ingemarsson, 2003, Kent et al., 2012, Peacock et al., 2012, Lawn and Kinney, 2014). Hence, the higher proportion of males in the Indigenous preterm groups may contribute to their higher incidence of renal injury and renal impairment, and this is an important area for future research. In addition, there was a much higher incidence of IUGR in the Indigenous infants in both the very and moderately preterm groups, which may have contributed to their heightened vulnerability to preterm birth. Certainly, there have been many studies that have reported an adverse impact of IUGR on nephrogenesis and nephron endowment (Hinchliffe et al., 1992, Manalich et al., 2000, Zohdi et al., 2012); hence, the IUGR

infants may already have compromised renal growth and function prior to their preterm delivery. Interestingly, in the extremely preterm category, the proportion of IUGR infants was less in the Indigenous cohort compared with the non-Indigenous cohort; it is possible that this may have been due to higher mortality in the IUGR Indigenous infants born extremely preterm compared with IUGR non-Indigenous infants, rather than the number of IUGR births *per se.*

CONCLUSION

The findings of this study highlight the vulnerability of Indigenous infants to renal impairment following preterm birth, and this is likely to adversely impact lifelong renal health. It is therefore imperative in future studies to elucidate the underlying mechanisms leading to renal vulnerability of Indigenous infants born preterm.

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STATEMENT OF COMPETING FINANCIAL INTERESTS

None

REFERENCES

AIHW 2011. Australia's welfare 2011. Australia's welfare no. 10. Cat. no. AUS 142. Canberra: AIHW.

AIHW 2014. Australia's health series no. 14. Cat. no. AUS 178. Canberra: AIHW.

AIHW 2015. Australia's welfare 2015. Australia's welfare no. 12. Cat. no. AUS 189. Canberra: AIHW.

ANZDATA 2015. Chapter 12: Indigenous People and End Stage Kidney Disease. Australia and New Zealand Dialysis and transplant Registry, Adelaide, Australia. *ANZDATA Registry. 37th Report*.

ANZDATA 2016. ANZDATA Registry. 38th Report, Chapter 1: Incidence of End Stage Kidney Disease. Australia and New Zealand Dialysis and Transplant Registry, Adelaide, Australia. 2016.

BECK, S., WOJDYLA, D., SAY, L., BETRAN, A. P., MERIALDI, M., REQUEJO, J. H., RUBENS, C., MENON, R. & VAN LOOK, P. F. 2010. The worldwide incidence of preterm birth: a systematic review of maternal mortality and morbidity. *Bull World Health Organ,* 88**,** 31-8.

BUEVA, A. & GUIGNARD, J. 1994. Renal function in the preterm neonate. *Pedatric Research,* 36**,** 572-577.

CASS, A., CUNNINGHAM, J., WANG, Z. & HOY, W. 2001. Regional variation in the incidence of end-stage renal disease in Indigenous Australians. *Med J Aust,* 175**,** 24-7.

CLARKE, M. & BOYLE, J. 2014. Antenatal care for Aboriginal and Torres Strait Islander women. *Australian Family Physician,* 43**,** 20-24.

CONTI, M., MOUTEREAU, S., ZATER, M., LALLALI, K., DURRBACH, A., MANIVET, P., ESCHWEGE, P. & LORIC, S. 2006. Urinary cystatin C as a specific marker of tubular dysfunction. *Clin Chem Lab Med,* 44**,** 288-91.

DUBOWITZ, L., DUBOWITZ, V. & GOLDBERG, C. 1970. Clinical assessment of gestational age in the newborn infant. *J Pediatr,* 77**,** 1-10.

FELL, J. M., THAKKAR, H., NEWMAN, D. J. & PRICE, C. P. 1997. Measurement of albumin and low molecular weight proteins in the urine of newborn infants using a cotton wool ball collection method. *Acta Paediatr,* 86**,** 518-22.

FONG, D., DENTON, K. M., MORITZ, K. M., EVANS, R. & SINGH, R. R. 2014. Compensatory responses to nephron deficiency: adaptive or maladaptive? *Nephrology (Carlton),* 19**,** 119-28.

GALLINI, F., MAGGIO, L., ROMAGNOLI, C., MARROCCO, G. & TORTOROLO, G. 2000. Progression of renal function in preterm neonates with gestational age < or = 32 weeks. *Pediatr Nephrol,* 15**,** 119-24.

GILBERT, T., CIBERT, C., MOREAU, E., GERAUD, G. & MERLET-BENICHOU, C. 1996. Early defect in branching morphogenesis of the ureteric bud in induced nephron deficit. *Kidney Int,* 50**,** 783-95.

GUBHAJU, L., SUTHERLAND, M. R., HORNE, R. S., MEDHURST, A., KENT, A. L., RAMSDEN, A., MOORE, L., SINGH, G., HOY, W. E. & BLACK, M. J. 2014. Assessment of renal functional maturation and injury in preterm neonates during the first month of life. *Am J Physiol Renal Physiol,* 307**,** F149-58.

HECKMANN, M., HARTMANN, M. F., KAMPSCHULTE, B., GACK, H., BODEKER, R. H., GORTNER, L. & WUDY, S. A. 2005. Assessing cortisol production in preterm infants: do not dispose of the nappies. *Pediatr Res,* 57**,** 412-8.

HINCHLIFFE, S. A., LYNCH, M. R., SARGENT, P. H., HOWARD, C. V. & VAN VELZEN, D. 1992. The effect of intrauterine growth retardation on the development of renal nephrons. *Br J Obstet Gynaecol,* 99**,** 296-301.

HOY, W. E., HUGHSON, M. D., SINGH, G. R., DOUGLAS-DENTON, R. & BERTRAM, J. F. 2006. Reduced nephron number and glomerulomegaly in Australian Aborigines: a group at high risk for renal disease and hypertension. *Kidney Int,* 70**,** 104-10.

HOY, W. E. & NICOL, J. L. 2010. Birthweight and natural deaths in a remote Australian Aboriginal community. *Med J Aust,* 192**,** 14-9.

INGEMARSSON, I. 2003. Gender aspects of preterm birth. *BJOG,* 110 Suppl 20**,** 34-8.

KENT, A. L., WRIGHT, I. M., ABDEL-LATIF, M. E., NEW SOUTH, W. & AUSTRALIAN CAPITAL TERRITORY NEONATAL INTENSIVE CARE UNITS AUDIT, G. 2012. Mortality and adverse neurologic outcomes are greater in preterm male infants. *Pediatrics,* 129**,** 124-31.

KJELDSEN, L., JOHNSEN, A. H., SENGELOV, H. & BORREGAARD, N. 1993. Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase. *J Biol Chem,* 268**,** 10425-32.

LAWN, J. E. & KINNEY, M. 2014. Preterm birth: now the leading cause of child death worldwide. *Sci Transl Med,* 6**,** 263ed21.

MANALICH, R., REYES, L., HERRERA, M., MELENDI, C. & FUNDORA, I. 2000. Relationship between weight at birth and the number and size of renal glomeruli in humans: a histomorphometric study. *Kidney Int,* 58**,** 770-3.

NAGAI, J. & TAKANO, M. 2004. Molecular aspects of renal handling of aminoglycosides and strategies for preventing the nephrotoxicity. *Drug Metab Pharmacokinet,* 19**,** 159-70.

NAUTA, F. L., BOERTIEN, W. E., BAKKER, S. J., VAN GOOR, H., VAN OEVEREN, W., DE JONG, P. E., BILO, H. & GANSEVOORT, R. T. 2011. Glomerular and tubular damage markers are elevated in patients with diabetes. *Diabetes Care,* 34**,** 975-81.

PEACOCK, P. J., HENDERSON, J., ODD, D. & EMOND, A. 2012. Early school attainment in late-preterm infants. *Arch Dis Child,* 97**,** 118-20.

PRESTON-THOMAS, A., CASS, A. & O'ROURKE, P. 2007. Trends in the incidence of treated end-stage kidney disease among Indigenous Australians and access to treatment. *Aust N Z J Public Health,* 31**,** 419-21.

SAYERS, S. M. & POWERS, J. R. 1993. Birth size of Australian aboriginal babies. *Med J Aust,* 159**,** 586-91.

SPENCER, J., WANG, Z. & HOY, W. E. 2001. Low birth weight and reduced renal volume in Aboriginal children. *American Journal of Kidney Diseases,* 37**,** 915-920.

TSUKAHARA, H., FUJII, Y., TSUCHIDA, S., HIRAOKA, M., MORIKAWA, K., HARUKI, S. & SUDO, M. 1994. Renal handling of albumin and beta-2-microglobulin in neonates. *Nephron,* 68**,** 212-6.

TSUKAHARA, H., YOSHIMOTO, M., SAITO, M., SAKAGUCHI, T., MITSUYOSHI, I., HAYASHI, S., NAKAMURA, K., KIKUCHI, K. & SUDO, M. 1990. Assessment of tubular function in neonates using urinary beta 2-microglobulin. *Pediatric Nephrology,* 4**,** 512-4.

TUKEY, J. W. 1977. Exploratory data analysis. *Addison-Wesley*.

WHITE, S. L., PERKOVIC, V., CASS, A., CHANG, C. L., POULTER, N. R., SPECTOR, T., HAYSOM, L., CRAIG, J. C., SALMI, I. A., CHADBAN, S. J. & HUXLEY, R. R. 2009. Is low birth weight an antecedent of CKD in later life? A systematic review of observational studies. *Am J Kidney Dis,* 54**,** 248-61.

YOU, J., HOY, W., ZHAO, Y., BEAVER, C. & EAGAR, K. 2002. End-stage renal disease in the Northern Territory: current and future treatment costs. *Med J Aust,* 176**,** 461-5.

ZOHDI, V., SUTHERLAND, M. R., LIM, K., GUBHAJU, L., ZIMANYI, M. A. & BLACK, M. J. 2012. Low Birth Weight due to Intrauterine Growth Restriction and/or Preterm Birth: Effects on Nephron Number and Long-Term Renal Health. *Int J Nephrol,* 2012**,** 136942.

FIGURES AND LEGENDS

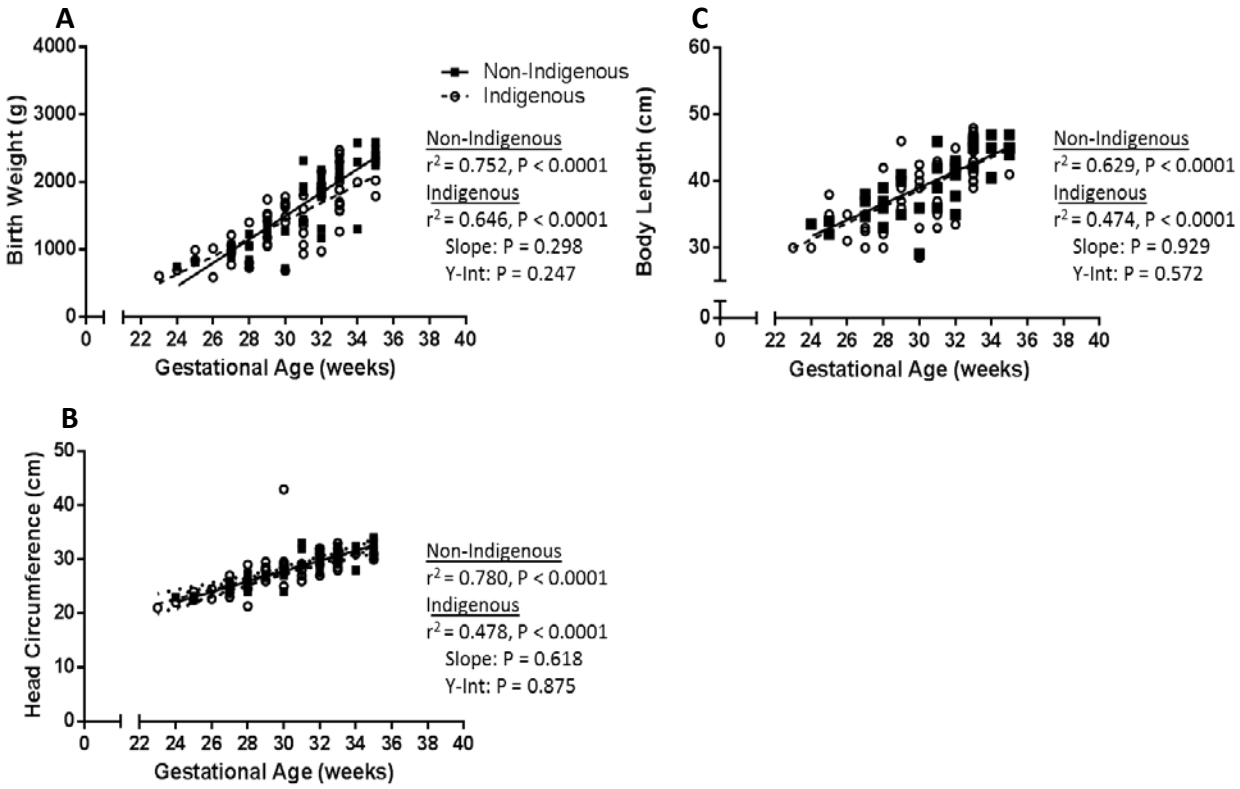


Figure 1. Birth growth of premature infants. Linear regression analyses of birth weight (A), head circumference (B), and body length (C) in all Indigenous (o) and non-Indigenous (■) infants relative to gestational age at the time of delivery.

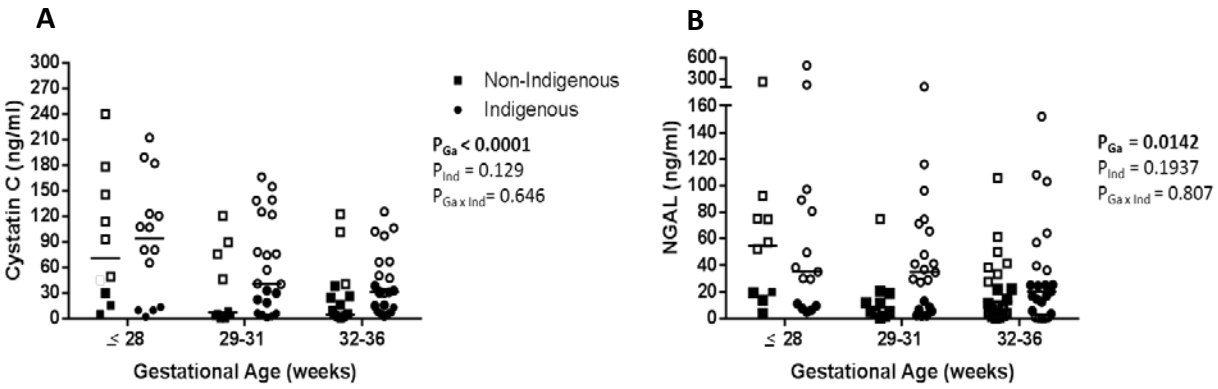


Figure 2. Renal injury markers cystatin-C and NGAL at day 4 of life. Median values for urine cystatin-C levels (A) and urine neutrophil gelatinase-associated lipocalin (NGAL) levels (B), in infants born extremely preterm (≤ 28 weeks of gestation (Indigenous (cystatin - C) n = 14 (NGAL) n = 15; non-Indigenous n = 10)), very preterm (29-31 weeks of gestation (Indigenous n = 21; non-Indigenous n = 10)), and moderately preterm (32- 36 weeks of gestation (Indigenous n = 23; non-Indigenous n = 22)), at day 4 of life. Data were analysed using a two-way ANOVA with the factors: effect of degree of prematurity ($P_{Ga;}$ extremely preterm *versus* very preterm *versus* moderately preterm), effect of Indigenous ethnicity ($P_{Ind;}$ indigenous *versus* non-Indigenous), and their interaction ($P_{Ga\ x\ Ind}$). Abnormally high levels of cystatin-C and NGAL in preterm infants (compared to term infants) were indicated by a (□) for non-Indigenous infants and (o) for Indigenous infants.

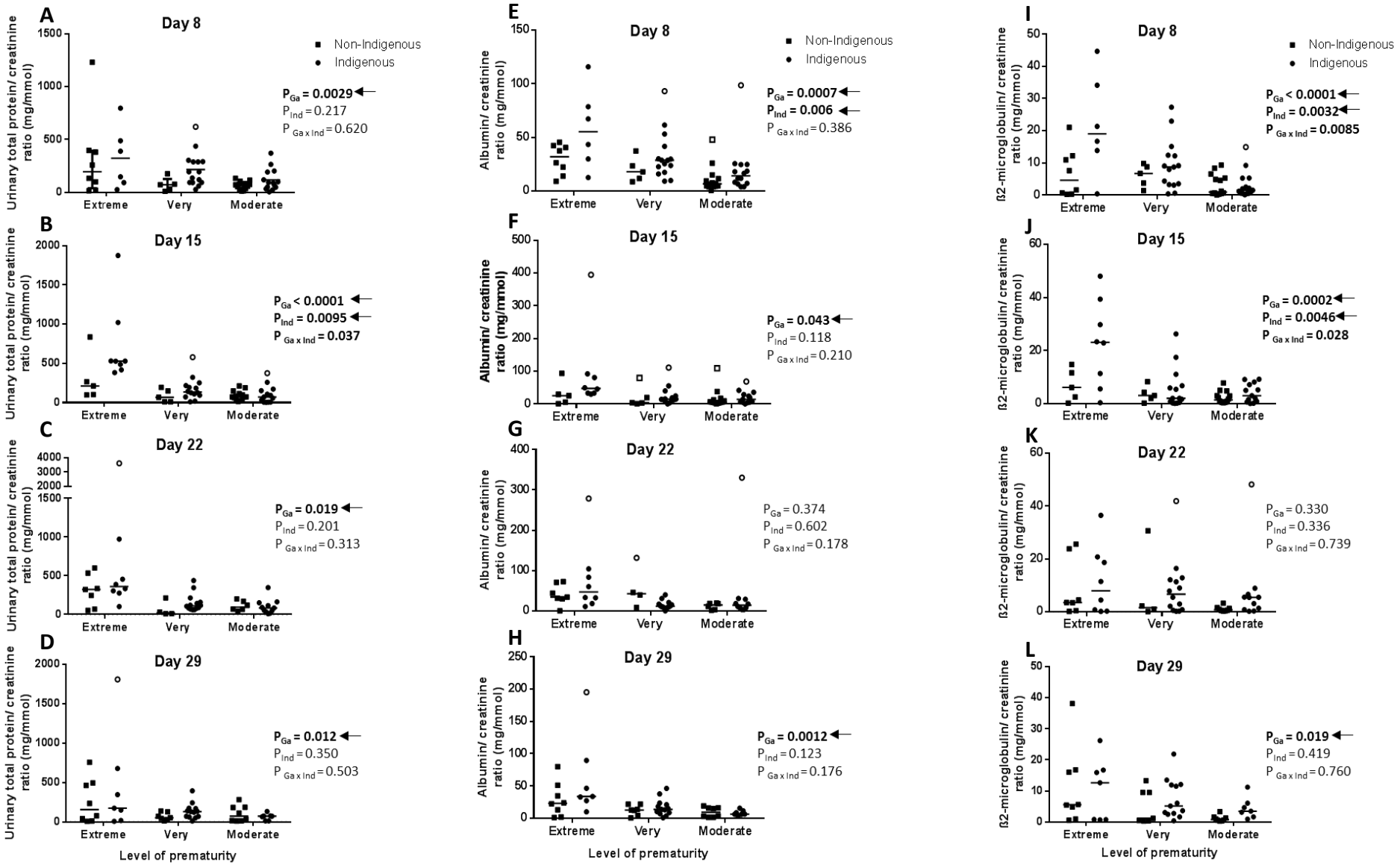
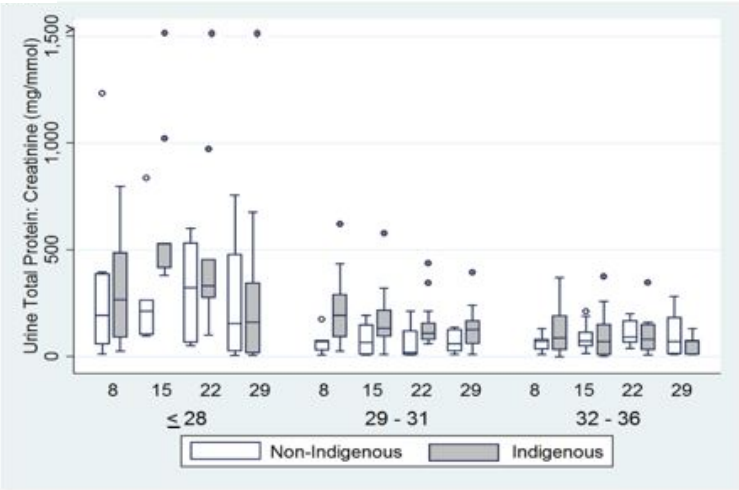
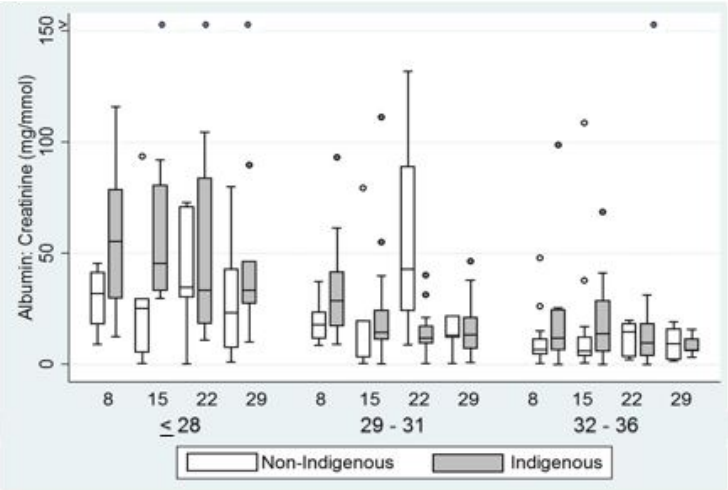

A
Day 8
Urinary total protein/ creatinine ratio (mg/mmol)
Non-Indigenous
Indigenous
P_{Ga} = 0.0029
P_{Ind} = 0.217
$P_{Ga \times Ind}$ = 0.620
B
Day 15
P_{Ga} < 0.0001
P_{Ind} = 0.0095
$P_{Ga \times Ind}$ = 0.037
C
Day 22
P_{Ga} = 0.019
P_{Ind} = 0.201
$P_{Ga \times Ind}$ = 0.313
D
Day 29
P_{Ga} = 0.012
P_{Ind} = 0.350
$P_{Ga \times Ind}$ = 0.503
Extreme
Very
Moderate
Level of prematurity
E
Day 8
Albumin/ creatinine ratio (mg/mmol)
P_{Ga} = 0.0007
P_{Ind} = 0.006
$P_{Ga \times Ind}$ = 0.386
F
Day 15
Albumin/ creatinine ratio (mg/mmol)
P_{Ga} = 0.043
P_{Ind} = 0.118
$P_{Ga \times Ind}$ = 0.210
G
Day 22
Albumin/ creatinine ratio (mg/mmol)
P_{Ga} = 0.374
P_{Ind} = 0.602
$P_{Ga \times Ind}$ = 0.178
H
Day 29
Albumin/ creatinine ratio (mg/mmol)
P_{Ga} = 0.0012
P_{Ind} = 0.123
$P_{Ga \times Ind}$ = 0.176
Extreme
Very
Moderate
Level of prematurity
I
Day 8
ß2-microglobulin/ creatinine ratio (mg/mmol)
Non-Indigenous
Indigenous
P_{Ga} < 0.0001
P_{Ind} = 0.0032
$P_{Ga \times Ind}$ = 0.0085
J
Day 15
ß2-microglobulin/ creatinine ratio (mg/mmol)
P_{Ga} = 0.0002
P_{Ind} = 0.0046
$P_{Ga \times Ind}$ = 0.028
K
Day 22
ß2-microglobulin/ creatinine ratio (mg/mmol)
P_{Ga} = 0.330
P_{Ind} = 0.336
$P_{Ga \times Ind}$ = 0.739
L
Day 29
ß2-microglobulin/ creatinine ratio (mg/mmol)
P_{Ga} = 0.019
P_{Ind} = 0.419
$P_{Ga \times Ind}$ = 0.760
Extreme
Very
Moderate
Level of prematurity

Figure 3. Effect of gestational age and Indigenous ethnicity on protein excretion over the first month of life. Scatterplot represents data from each individual infant. The median values (50%), are shown by the line within each prematurity group. The indigenous infants are represented by the circle symbols and the non-Indigenous infants are represented by the square symbols. The open circles represent the abnormal values (above 1.5 times the 75th% confidence interval from the median) for urine total protein/creatinine (A-D), albumin/creatinine (E-H) and β2-microglobulin/creatinine (I-L) levels corrected for urine creatinine, on postnatal days 8, 15, 22 and 29 in preterm infants. Extremely preterm infants ($\leq$ 28 weeks' gestation), very preterm infants (29 - 31 weeks' gestation) and moderately preterm infants (32 - 36 weeks' gestation). Data were analysed using a two-way ANOVA with the factors: effect of degree of prematurity ($P_{Ga;}$ extremely preterm *versus* very preterm *versus* moderately preterm), effect of Indigenous ethnicity ($P_{Ind;}$ indigenous *versus* non-Indigenous), and their interaction ($P_{Ga \times Ind}$).

A


Urine Total Protein: Creatinine (mg/mmol)
0
500
1,000
1,500+
8 15 22 29 8 15 22 29 8 15 22 29
≤ 28
29 - 31
32 - 36
Non-Indigenous
Indigenous

B


Albumin: Creatinine (mg/mmol)
0
50
100
150+
8 15 22 29 8 15 22 29 8 15 22 29
≤ 28
29 - 31
32 - 36
Non-Indigenous
Indigenous

C

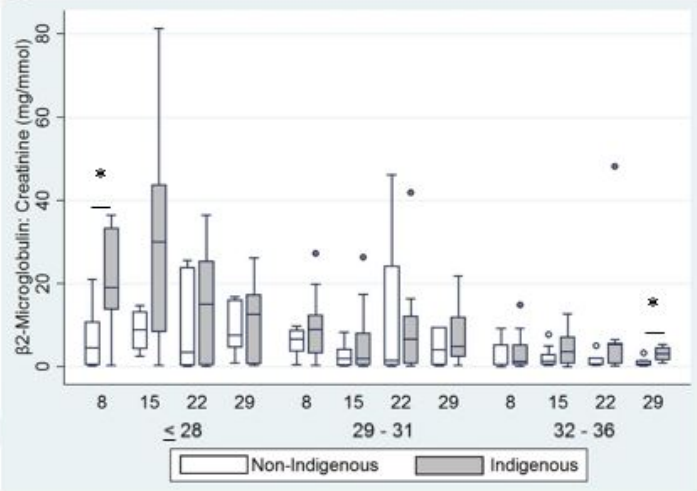

β2-Microglobulin: Creatinine (mg/mmol)
0
20
40
60
80
8 15 22 29 8 15 22 29 8 15 22 29
≤ 28
29 - 31
32 - 36
Non-Indigenous
Indigenous

Figure 4. Effect of postnatal age and Indigenous ethnicity on protein excretion over the first month of life. Box plots represent the interquartile ranges (IQR: 25th and 75th percentiles) and median (50%), with whiskers extending to 1.5 times the IQR from the box edges. Outliers are represented by (●) for urine total protein/creatinine (A), albumin/creatinine (B) and β2-microglobulin/creatinine (C), on postnatal days 8, 15, 22 and 29 in preterm infants. Extremely preterm infants (≤ 28 weeks' gestation), very preterm infants (29 - 31 weeks' gestation) and moderately preterm infants (32 - 36 weeks' gestation). *P < 0.05 Indigenous *versus* non-Indigenous infants within the gestational age group.

Table 1. Infant demographics. The median [Interquartile range] and mean ± SD of body parameters including: birth weight, body length, and head circumference in extremely preterm (≤ 28 weeks' gestation), very preterm (29 - 31 weeks' gestation), and moderately preterm (32-36 weeks' gestation) Indigenous and non-Indigenous infants. The percentage of males and intrauterine growth restricted (IUGR) infants in each gestational age and ethnicity group is also shown. *P < 0.05 Indigenous *versus* non-Indigenous within gestational age groups.

	Birth Weight (g)	Body Length (cm)	Head Circumference (cm)	Male (%)	IUGR (%)
EXTREMELY PRETERM					
Non-Indigenous (n = 11) Median (min, max) Mean ± SD	883 (742, 1424) 964.82 ± 214.77	35 (32, 39) 35.29 ± 2.11	24 (22.5, 27.3) 24.61 ± 1.61	**55%** (6/11)	**27%** (3/11)
Indigenous (n = 17) Median (min, max) Mean ± SD	987 (585, 1426) 986.17 ± 236.46	35 (30, 42) 34.97 ± 3.76	24.5 (21.3, 29) 24.81 ± 2.25	**76%** (13/17)	**18%** (3/17)
VERY PRETERM					
Non-Indigenous (n = 10) Median (min, max) Mean ± SD	1446 (710, 2320) 2076.6 ± 331.46	40.5 (29, 46) 39.5 ± 5.15	28 (24, 33) 28.56 ± 3.02	**40%** (4/10)	**10%** (1/10)
Indigenous (n = 20) Median (min, max) Mean ± SD	1491 (685, 1802) 1435.35 ± 303.95	39 (28.5, 46) 38.42 ± 4.11	29.3 (25, 43) 28.89 ± 3.74	**60%** (12/20)	**30%** (6/20)
MODERATELY PRETERM					
Non-Indigenous (n = 22) Median (min, max) Mean ± SD	2245 (1167, 2590) 2109.19 ± 409.82	45 (35, 47) 43.44 ± 3.08	31.5 (27.5, 34) 31.24 ± 1.70	**45%** (10/22)	**9%** (2/22)
Indigenous (n = 23) Median (min, max) Mean ± SD	*1887 (605, 2470) 1813.5 ± 417.13	*42.4 (30, 48) 42.14 ± 4.08	30.35 (21, 33) 29.94 ± 2.34	**67%** (16/23)	**25%** (6/23)

Table 2: Measures of renal impairment: Measures of renal impairment in 17 preterm infants that exhibited $\geq$ 2 measures at one or more time points, grouped by gestational age and ethnicity. Table shows each infant's characteristics including gender (M is male; F is female), whether they were intrauterine growth restricted (IUGR; shaded), and the postnatal day that the infant exhibited the $\geq$ 2 measures of renal impairment. Protein levels that were considered abnormally high for each time point are shaded and indicated by an X.

Infant	Sex	IUGR	High Cystatin-C (Postnatal Day 4)	High NGAL (Postnatal Day 4)	Postnatal Days	Pathological Proteinuria	High Urine Total Protein	High Albumin	High β2-Microglobulin
EXTREMELY PRETERM INFANTS									
Indigenous (n = 3)									
1	M		X	X	**15**	X	X	X	
2	M				**22**	X	X	X	
3	F		X		**22**	X			
			X		**29**	X		X	
Non-Indigenous (n = 3)									
1	M		X	X	**8**	X			
2	F		X		**8**	X			
3	F		X	X	**15**	X			
VERY PRETERM INANTS									
Indigenous (n = 7)									
1	M		X	X	**8**	X			
2	M			X	**8**		X		
3	F			X	**8**	X			
4	F			X	**8**	X		X	
5	F			X	**15**			X	
6	M		X		**15**	X	X		
7	F		X	X	**22**				X
MODERATELY PRETERM INFANTS									
Indigenous (n = 4)									
1	F			X	**8**				X
2	F			X	**15**		X		
				X	**22**		X		
3	F		X		**15**			X	
4	M		X		**22**			X	X

NGAL – urinary neutrophil gelatinase associated lipocalin, IUGR – intrauterine growth restriction, M – male, F – female.

Chapter Seven:

DISCUSSION AND CONCLUSION

7. DISCUSSION AND CONCLUSIONS

The findings of this thesis provide important insight into the normal development of the human kidney and the impact of intrauterine growth restriction (IUGR). The findings suggest that during normal development there are intrinsic mechanisms within the kidneys of all individuals that control the pace of nephrogenesis, but ultimately lead to a similar number of glomerular generations being formed within the kidney. In addition, the relative proportions of the different glomerular cell types appears to remain relatively constant once glomeruli have developed into a mature form, such that the cellular composition is similar in developed glomeruli at mid-gestation and at term. Importantly, the findings show that IUGR adversely impacts nephrogenesis, leading to a reduction in the number of glomerular generations formed within the kidneys at birth and subsequently rendering a vulnerability to renal dysfunction in infants born preterm. Aboriginal ethnicity also rendered susceptibility to renal impairment following preterm birth. My studies conducted using a lamb model support previous findings of renal and glomerular hypertrophy following preterm birth, and show that preterm lambs that were ventilated after birth have reduced glomerular capillary growth compared to lambs born at term. Overall, my findings highlight many directions for future research.

7.1 RENAL DEVELOPMENT FROM MID TO LATE GESTATION IN MALE AND FEMALE FETUSES

In order to get an understanding of how preterm birth affects the developing kidneys, it was considered important in the first experimental chapter of this of this thesis (chapter 2) to examine understanding of normal kidney development and nephrogenesis during late gestation, at the time when preterm birth occurs. Indeed, as shown in Figure 7.1, the second half of gestation is the critical period for nephrogenesis in the human kidney, with the majority

of nephrons formed during this period. Nephrogenesis commences at around 5 weeks of gestation (Osathanondh and Potter, 1963); at 9 weeks of gestation the first nephrons begin to form (Woolf et al., 2003, Cullen-McEwen et al., 2016) and during the final trimester of pregnancy approximately 60% of the nephrons are formed (Hinchliffe et al., 1991).

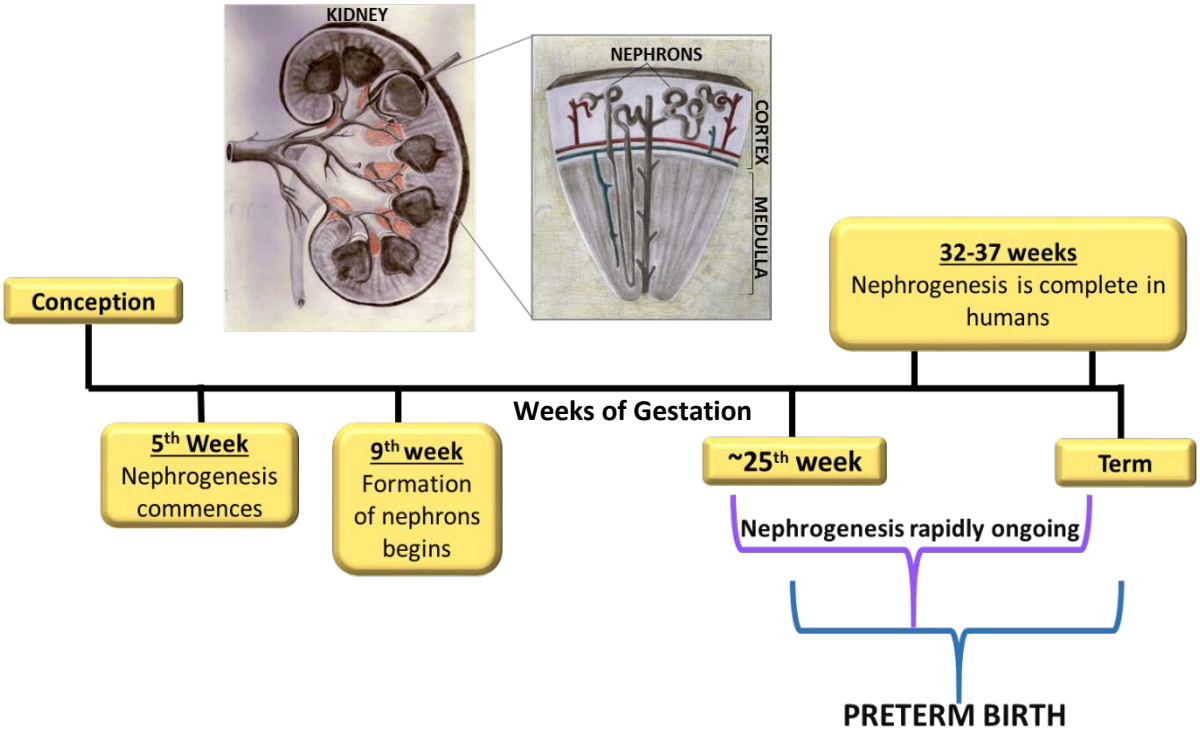


Figure 7.1. Timeline of nephrogenesis. Nephrogenesis commences in early gestation (~5th week of gestation), and the first nephrons are formed at 9 weeks of gestation. The majority of nephrons are formed during the third trimester of pregnancy, which coincides with the time when preterm birth occurs. Figure adapted from Black et al., (2011).

When conducting my studies, the human fetal kidneys to be analysed were carefully selected based on the autopsy reports, such that only the kidneys from infants that were appropriately grown and died suddenly *in utero* were analysed. Importantly, the findings of my studies highlight the temporal variability in nephrogenesis within normally grown human kidneys, which up until now has not been fully appreciated. The findings highlight the wide variation in the timing of the cessation of nephrogenesis, ranging from 33 weeks of gestation to later than 37 weeks' gestation. Interestingly, infants that had an early cessation of nephrogenesis (~33 weeks' gestation) exhibited a similar number of glomerular generations within the renal cortex as infants that had a later cessation of nephrogenesis, and term infants. Given that the number of glomerular generations correlates with nephron number (Hinchliffe et al., 1991), my findings suggest that the number of nephrons formed within the kidney is not adversely impacted upon by the early cessation of nephrogenesis. My findings also support the idea that there are intrinsic mechanisms within the kidneys of all individuals that control the pace of nephrogenesis, but ultimately lead to a similar number of glomerular generations being formed. Hence, the large variability in nephron number in the adult population (ranging from approximately 250,000 nephrons to 2.5 million nephrons (Cullen-McEwen et al., 2016) does not appear to be due to innate variability in the number of glomerular generations formed within the kidneys during normal renal development.

As a follow-up to my findings, it is now important to conduct prospective studies (whereby precise fractions of the kidneys can be collected at autopsy) in order to compare the overall variability in the number of nephrons formed within developing human kidneys. Given that the number of glomerular generations within the kidneys are similar, the question arises as to whether the total number of nephrons formed within appropriately grown human kidneys are also within a similar range. Certainly, the total number of nephrons formed within other species

appears to be relatively tightly regulated; for example, in rats and mice there is approximately 29% variability in nephron number at the time of birth (Cullen et al. 2016). However, unlike rodents that have only one renal papilla, this may not be the case in human kidneys given that there is wide variation in the number of papillae formed (ranging from 4 to 18) (Treuting and Kowalewska, 2011). This is likely due to early differences in branching morphogenesis of the ureteric duct early in gestation. It is likely that, even if the number of glomerular generations is relatively constant, the number of nephrons will be significantly increased in kidneys with high numbers of papillae compared to kidneys with a low number of papillae. In future stereological analyses of nephron number in developing fetal kidneys, it will be important to examine the number of nephrons formed within the kidneys relative to the number of renal papillae.

Furthermore, as a follow-up to my findings it is also important to investigate how genes implicated in branching can potentially modify the number of glomerular generations formed within the kidney. In addition, in future studies it is essential to assess the expression of renal progenitor cells during kidney development such as the receptor tyrosine kinase RET, its ligand glial cell-line derived neurotrophic factor (GDNF) and paired box 2 (PAX2), which all determine ureteric cell fate (ureteric bud branching) (Shakya et al., 2005). Previous mice studies have shown that a reduction in progenitor cells during fetal development leads to renal hypoplasia and low nephron number at birth (Cain et al., 2010, Cebrian et al., 2014), where downregulation of GDNF/RET signaling caused reduced nephron endowment (approximately 30%) in mice heterozygous for GDNF (Cullen-McEwen et al., 2001). Likewise, the presence of known polymorphisms of RET (1476A) and PAX2 (AAA haplotype), resulted in ~10% reduction in kidney volume/body surface area, proportional to reduced nephron number at birth (Quinlan et al., 2007, Zhang et al., 2008). Moreover, mutations in PAX2 have been shown to lead to oligomeganephronia (reduced number of lobes in kidney) (Salomon et al., 2001).

7.1.1 DIFFERENCES IN THE GROWTH OF GLOMERULI BETWEEN SEXES

Another interesting finding in my studies of human fetal renal development was the apparent sexual dimorphism in the size of glomeruli over the second half of gestation. In female infants there was a positive correlation between glomerular cross-sectional area and gestational age, but this was not evident in the kidneys of male infants, where glomerular cross-sectional area remained relatively consistent over gestation. Prior to my study, there have only been a small number of studies that have looked at glomerular size during late gestational development in humans. In one study, 69 fetal kidneys were examined (Souster and Emery, 1980), and it was found that the size of glomeruli (as assessed by measuring mean glomerular area) remained constant from 20 weeks' gestation until term. Conversely, in another study of 86 fetuses Ferraz et al., (2008), it was reported that the size of glomeruli (as assessed by measuring the area and diameter of the glomerular tuft and renal corpuscle) increased from 15 weeks' gestation to term. However, neither of these studies separately analysed the kidneys from male and female infants. Indeed, my findings would suggest that there may have been more male infants included in the first study and more females included in the second study. Overall, my findings highlight the importance of considering the sex of the infant when assessing glomerular size in future studies.

Interestingly, when I examined glomerular cross-sectional area in the kidneys of IUGR infants the sexual dimorphism in relation to glomerular size remained; however, the overall effects were different to that observed in the appropriately grown kidneys, with an apparent adverse impact of IUGR. In contrast, to appropriately grown infants, the growth of glomeruli in female IUGR infants remained constant across gestation, whereas in males the glomerular size decreased over gestation. It is important to note that there are limitations with these findings, given that glomerular size was assessed by measuring glomerular cross-sectional area; this

measurement is affected by the plane at which the section is taken through the glomeruli. However, given that at least 100 glomeruli were measured per kidney, we are confident that our measurements provide a relatively accurate assessment of glomerular size; however, in future studies it would be beneficial to confirm our findings by measuring glomerular volume.

7.1.2 CELLULAR COMPOSITION OF GLOMERULI IN DEVELOPING HUMAN KIDNEYS

Interestingly, in glomeruli that were considered mature at the time of analysis, the proportion of the different glomerular cell types (podocytes, endothelial cells, and mesangial cells) within the glomeruli remained relatively constant over the second half of gestation, even in the kidneys of normally grown female infants, where there was an apparent increase in glomerular cross-sectional area over the late gestational period. Hence, it is plausible to suggest that once glomeruli reach full maturity *in utero*, whether it is mid or late in gestation, the number of glomerular cells remain constant until birth. Furthermore, the relative glomerular cellular composition does not appear to be different between the kidneys of males and females. Prior to the studies in this thesis (chapters 2 and 4), there had been no studies that had looked at the cellular composition of glomeruli in the developing human kidneys. Interestingly, studies in child and adult kidneys (Puelles et al., 2015) have reported increasing numbers of podocytes and non-podocyte cells with increasing glomerular size, and the absolute number of the relative cell types reported were much higher than I found in the fetal kidneys. This is intriguing given that podocytes are considered to cease proliferating prior to birth. Hence, in future studies it is important to gain insight into the mechanisms relating to the changes in the cellular composition of glomeruli in postnatal life. Indeed, it is popular opinion that the parietal cells of the Bowman's capsule can migrate into glomeruli postnatally, and subsequently differentiate into podocytes (Lasagni and Romagnani, 2010, Lazzeri et al., 2010, Shankland et al., 2013).

In regards to glomerular endothelial cell number, my studies in chapter 3 clearly show that as a result of being born that there is marked growth of the glomerular capillaries postnatally. This is likely mediated by endothelial proliferation and it is important to verify this in future studies. Of concern, however, my studies (chapter 3) show that mechanical ventilation adversely impacts glomerular capillary growth. Mechanical ventilation in term lambs led to reduced growth of capillaries compared to non-ventilated lambs. Importantly, ventilation in preterm lambs (which is the scenario most common in the clinical setting) led to a significant reduction in total renal filtration surface area when compared to term non-ventilated lambs. The reduced growth of capillaries did not appear to be mediated by the downregulation of VEGF expression at approximately 72 hours after birth (time of necropsy), however it is possible that a reduction in VEGF expression may have occurred acutely when the lambs were initially ventilated.

7.2 ANTECEDENTS OF PRETERM BIRTH, SPECIFICALLY INTRAUTERINE GROWTH RESTRICTION, HAVE THE POTENTIAL TO ADVERSELY IMPACT NEPHROGENESIS AND RENDER THE KIDNEYS VULNERABLE TO PRETERM BIRTH

IUGR is a common antecedent of preterm birth and hence is a common comorbidity in preterm infants. One of the major causes of IUGR is placental insufficiency which leads to *in utero* stressors such as malnutrition and/or oxygen deprivation in the developing fetus. It is plausible that *in utero* stressors such as IUGR can have adverse impacts on the mechanisms involved in ureteric bud branching, and the ability of the mesenchymal cells to differentiate, during the time of rapidly ongoing nephrogenesis; each of these two processes are crucial for the determination of final nephron number (Moritz and Cullen-McEwen, 2006).

Certainly, many animal models have highlighted the adverse effects of IUGR on kidney size, nephron number and renal function *reviewed in* Zohdi et al., (2012), in both early life (Langley-

Evans et al., 1999, Mitchell et al., 2004, Zohdi et al., 2007) and more long-term (Langley-Evans et al., 1999, Zimanyi et al., 2004, Woods et al., 2004, Sahajpal and Ashton, 2005, Zimanyi et al., 2006, Hoppe et al., 2007, Wlodek et al., 2008, Moritz et al., 2009). However, to date only two studies have examined the effect of IUGR on nephrogenesis on the developing human kidneys (Hinchliffe et al., 1992, Manalich et al., 2000), and in these studies, only a small number of fetuses were analysed. For these reasons, a major aim of chapter 5 of this thesis was to comprehensively characterise the effects of IUGR on nephrogenesis in the developing human kidney, at a time when nephrogenesis was rapidly ongoing, and assess the impact of IUGR on renal growth.

Importantly, my findings show that although the number of glomerular generations formed within the kidneys in appropriately grown infants appears to remain relatively constant during normal fetal growth (chapter 2), this is not the case in IUGR infants. This is consistent with previous studies that have demonstrated that developmental insults during gestation have the potential to adversely impact nephrogenesis, and thus alter the normal trajectory of renal development (Dorey et al., 2014). Indeed, my findings clearly show that the number of glomerular generations formed within the kidneys of infants that were growth restricted *in utero* is significantly reduced when compared to appropriately grown infants *in utero,* and support the previous findings in human infants (where only a small number of infants were studied (Hinchliffe et al., 1992, Manalich et al., 2000). A particularly interesting finding of this thesis was the attenuated glomerular growth in response to growth restriction *in utero*. In IUGR kidneys, the growth of glomeruli appeared to be attenuated from ~25 weeks of gestation in both male and female fetuses; overall, males were more severely affected by IUGR than females (as described above in section 7.1.1).

In addition to the adverse effects of IUGR on renal morphology, there have been a multitude of epidemiological studies linking low birth weight (which can originate from IUGR and/or preterm birth) with adult renal disease (White et al., 2009). A meta-analysis of 31 of these studies reported a 70% increased risk of developing chronic renal disease in subjects born of low birth weight (White et al., 2009). My findings, and those of others, support the concept that this association between low birth weight and later renal disease relates to a reduced nephron endowment at the beginning of life in subjects born IUGR. Indeed, given that renal disease ultimately occurs as a result of a loss of functioning nephrons, it is expected that when renal pathologies begin to develop postnatally that the onset of renal dysfunction will be both accelerated and exacerbated when nephron number is already low at the time of disease onset. In the case of the preterm infant, the finding of impaired nephrogenesis as a result of IUGR is very important given that IUGR is a common antecedent and subsequent co-morbidity of preterm birth. Hence, in order to in future improve the short-term (and potentially long-term) renal health in these individuals, it was considered essential in the second series of experiments in chapter 5 to gain an understanding of how preterm birth combined with IUGR affects renal development and function in the neonatal period.

Surprisingly, at the end of the first week of life, ultrasound measurements of kidney size showed no difference in kidney growth between IUGR and appropriately grown infants. This was unexpected as my studies in chapter 5 showed that IUGR fetuses exhibited a marked reduction in renal growth during late gestation, and kidneys from the IUGR infants were significantly smaller at term. Furthermore, previous studies of renal growth *in utero* have shown slower kidney growth (measured using ultrasound and MRI) from 24 -34 weeks of gestation in IUGR fetuses, compared to appropriately grown fetuses (Konje et al., 1996). In addition, Silver et al. (2003) reported a 31 % decrease in renal volume (measured using ultrasonography) from 27 to

41 weeks of gestation, compared to appropriately grown fetuses. Although no differences were observed in kidney size in IUGR preterm infants in the first week of life in our study, reductions in kidney size did become apparent by the end of the first month; at that stage, IUGR infants born very or moderately preterm showed markedly reduced kidney length, width, depth and volume (specifically in the right kidney) compared to non-IUGR infants of the same age. One possible explanation for the similar kidney size measurements between the preterm IUGR and appropriately grown infants at day 8 of life, may relate to the renal hypertrophy that takes place following preterm birth, likely due to the increased renal blood flow and functional demands of the kidneys following birth. Since the fastest rate of renal growth is said to occur during the first few weeks of life (Zerin and Meyer, 2000) it is possible that the added renal hypertrophy as a result of preterm birth disguises any attenuation of kidney size during this early postnatal period of rapid kidney growth. In future, it would be interesting to conduct much longer follow up studies of IUGR and non-IUGR preterm infants to compare the long-term growth of the kidneys. Indeed, studies from other investigators have shown IUGR to result in smaller kidney volumes at 12 years of age compared to children born at an appropriate for gestational age (Rakow et al., 2008); however, once kidney volume was adjusted for body weight, gender and age, there were no significant difference in kidney size between groups.

The findings from this renal ultrasound study also suggest that the right kidney may be more vulnerable to IUGR than the left. In support of this finding, there are a number of lines of evidence that suggest that there is enhanced growth in the left kidney relative to right kidney (Emamian et al., 1993), with an enhanced renal artery blood supply in the left kidney compared to the right (Satyapal et al., 2003). Retrospectively, we could not address this in our autopsy studies, as only the left kidneys were analysed.

In addition to reduced kidney growth, IUGR infants born preterm exhibited an increased vulnerability to renal injury at the end of the first week of life. Of particular concern, preterm IUGR infants appeared to suffer a greater degree of tubular injury compared to non-IUGR preterm infants over the first month of life, as evidenced by significantly elevated excretion of ß2-microglobulin at days 8, 15 and 22 of life. Why the IUGR infants are more vulnerable to tubular injury is currently unknown and this is an important area for future research. Although there are many studies describing elevated blood pressure and impaired renal function in preterm infants (many of whom would have been IUGR) and low birth weight infants (many of whom would have been born preterm) it is now essential in future studies to differentiate the independent effects of IUGR and preterm birth on long-term renal health.

Although nephron deficits do not necessarily lead to overt renal dysfunction at the start of life, there is considerable evidence to suggest that a reduced nephron endowment can lead to increased risk of developing renal disease later in life (Hoy et al., 2005, Keijzer-Veen et al., 2005 , Ingelfinger, 2008). Collectively, my findings from chapter 5 support the concept that IUGR combined with preterm birth renders a vulnerability to the impaired growth and function of the kidneys in early postnatal life and this potentially may lead to life-long adverse repercussions to renal health. Such findings are of the utmost clinical importance, given that the neonatal period provides a critical window for intervention in preterm neonates, especially in those for whom nephrogenesis is ongoing at the time of birth. Together these findings suggest further avenues for research. For example, it would be interesting to assess gene expression of Wilms' tumor 1 (WT1) in the kidneys of IUGR infants in conjunction with the estimation of nephron number, as well as the cellular composition of glomeruli (number of podocyte, endothelial and mesangial cells). It is well known that GDNF/RET signaling is responsible for maintaining WT1 expression. WT1 also regulates ureteric branching

morphogenesis (Majumdar et al., 2003) and in IUGR fetuses born from mothers with malnutrition, downregulation of WT1 caused reduced nephron endowment at birth (Majumdar et al., 2003). This suggests that dysregulation of WT1 expression during fetal development may lead to altered nephrogenesis. Additionally, follow-up long term studies have shown persistent WT1 dysregulation which caused alterations in podocyte structure, function and gene expression (Majumdar et al., 2003). To further assess the extent of glomerular injury, using immunohistochemistry techniques, it would be good to test markers of apoptosis (activated caspase-3) (Yang et al., 2001) and proliferation (PCNA or Ki-67) (Nadasdy et al., 1994) of renal glomerular cells in IUGR infants with reduced nephron endowment.

7.3 POTENTIAL FOR LONG-TERM PROGRAMMING OF RENAL DISEASE AS A RESULT OF PRETERM BIRTH –PARTICULARLY IN INDIGENOUS AUSTRALIANS

In Australia, the most common causes of chronic kidney disease are diabetes, followed by glomerulonephritis (disease of inflammation of the glomeruli) and hypertension (Stumpers and Thomson, 2013). The prevalence of chronic kidney disease and end-stage kidney disease is significantly higher amongst Indigenous Australians in comparison to the non-Indigenous population (Stumpers and Thomson, 2013, AIHW, 2015). Importantly, meta-analyses have shown that low birth weight is linked to renal impairment in adulthood (White et al., 2009), which is particularly relevant to Indigenous Australians given their high rates of low birth weight (due to IUGR, preterm birth or their combination) (Hoy et al., 1998, Hoy et al., 1999). The incidence of low birth weight amongst Indigenous Australians is twice as high (~12%) as non-Indigenous Australians (6%) (Li et al., 2012), and preterm birth rates are also significantly higher amongst Indigenous Australians (13.5%) compared to non-Indigenous Australians (~8%) (Li et al., 2012). In autopsied adult kidneys, Hoy and colleagues (2006) observed a 30% decrease in nephron number in Indigenous Australians compared to non-Indigenous Australians,

accompanied by 27% larger glomerular volumes. Furthermore, in Indigenous Australian children, low birth weight was associated with increased rates of pathological albuminuria compared to children born with a normal birth weight (Singh and Hoy, 2004). Collectively, the findings strongly support the idea that the antecedents of chronic kidney disease and end stage kidney disease originate very early in life in Indigenous Australians (Hoy et al. 2006).

Indeed, the findings in this thesis (chapter 6) also support this concept. In chapter 6, I compared markers of renal injury in the immediate period after birth (at postnatal day 4) and after the first month of life (postnatal day 29) in Indigenous and non-Indigenous Australian preterm and term infants. Importantly, my findings showed that infants born to Indigenous mothers had an increased risk of renal impairment following preterm birth; in particular, renal tubular impairment which was evidenced by persistently high levels of β2-microglobulin compared to non-Indigenous infants, in all of the gestational age groups. Overall, ~13% of Indigenous preterm infants exhibited proteinuria in the first month of life, compared to ~4% of the non-Indigenous preterm infants, with pathological proteinuria also most commonly evident in the Indigenous infants born extremely and very preterm. Furthermore, the majority of infants considered at 'high risk' of developing renal dysfunction, in all gestational age groups, were identified as Indigenous. At 72 hours after birth, Indigenous infants also exhibited elevated cystatin-c and NGAL levels compared to non-Indigenous infants in all gestational age groups. Urinary cystatin-C and NGAL are known biomarkers for acute kidney injury in children and adults, and are predictors of renal impairment in adulthood (Conti et al., 2006, Bolignano et al., 2009). In my study, although both renal injury markers were elevated in infants with suspected renal injury, neither cystatin-C nor NGAL proved to be good predictors of the infants that would go on to develop pathological proteinuria in early postnatal life. In this regard, given the high prevalence of renal impairment in the Indigenous preterm infants, it is important in future

studies to focus on identifying robust biomarkers of early renal injury. If early therapeutic intervention could be implemented in these infants, it has the potential to not only improve their short-term renal function but also their life-long renal health.

Why the Indigenous preterm infants are more vulnerable to renal impairment is currently unknown. Certainly, given my findings in chapter 5, the high incidence of IUGR in the Indigenous preterm infants is a likely contributing factor to their greater renal impairment compared to the non-Indigenous preterm infants. In addition, amongst the Indigenous preterm infants, there was a higher proportion of males compared to the non-Indigenous infants, in both the very, and moderately preterm categories. In this regard, the concept of a 'male disadvantage' has been well recognised in regards to premature birth; preterm male infants have increased morbidity (such as sepsis, jaundice and respiratory complications) immediately after birth (Ingemarsson, 2003, Peacock et al., 2012, Tundidor et al., 2012, Lawn and Kinney, 2014), as well as increased long-term morbidity and overall mortality rates (Kent et al., 2012) in comparison to female preterm infants of the same gestational age at birth. Interestingly, however, previous studies in Indigenous Australian adults have noted higher rates of chronic kidney disease amongst females compared to males (*reviewed* in Hoy et al, (2012)) as well as ~5% lower nephron numbers compared to males (Hoy et al., 2006). Hence, these findings in Indigenous adults do not support a long-term male disadvantage in relation to renal function. However, increased abdominal adiposity and diabetes rates amongst Indigenous women are considered to be major contributors to the elevated chronic kidney disease in Indigenous women (Stumpers and Thomson, 2013), which may override any early life male disadvantage. In future studies, it is important to further explore the effects of sex on renal function in the preterm neonate. Indeed, my studies of the developing kidneys certainly do show differences

between the sexes in relation to glomerular growth during late gestation, both in kidneys from normally grown infants (chapter 2) and kidneys from IUGR infants (chapter 5).

There remains the possibility that there are innate genetic differences in Indigenous Australians that renders the kidneys more vulnerable to renal dysfunction following preterm birth. Indeed, up until recently Indigenous Australians have lived a nomadic hunter/gatherer life-style, and hence it is likely that there will be evolutionary genetic differences in renal function when compared to non-Indigenous Australians. To address this, it is important to conduct DNA studies from Indigenous and non-Indigenous preterm infants whereby potential genetic differences can be investigated. Important relevant genes to be investigated include genetic polymorphisms of PAX2, angiotensin-converting enzyme (ACE) rs4646994 polymorphism, and TP53 codon 72 polymorphism as these polymorphisms are associated with reduced kidney size (~8%) (Kaczmarczyk et al., 2013). Other genes specifically associated with susceptibility to kidney injury (du Cheyron et al., 2008) and increased tubular injury (signified by elevated albumin levels) in Indigenous Australians (Duffy et al., 2016) should be investigated. Concomitantly, in order to quantify the extent of renal injury, future studies could also examine additional markers of renal tubular injury, such as kidney injury molecule-1 (KIM-1) (Vaidya et al., 2006).

Alternatively, there are many factors during pregnancy (including intrauterine infection and inflammation, maternal diabetes, antenatal medications, oligohydramnios) that may impact on renal development of the fetus and subsequently render the kidneys vulnerable to preterm birth. How these factors impact kidney development and function are likely applicable to pregnancies of all ethnicities; however, their prevalence may differ between different ethnic backgrounds. In the following sections the impact of many of these factors on renal

development are described, and they highlight the importance in future studies to examine the impact of these factors when combined with preterm birth.

7.4 INTRAUTERINE FACTORS THAT CAN POTENTIALLY IMPACT THE DEVELOPMENT OF THE IMMATURE KIDNEYS – IMPORTANT AREAS FOR FUTURE RESEARCH

It is now well recognised that the *in utero* environment can directly influence fetal organ development and structure. Hence, it is likely that the factors that lead to the induction of preterm delivery (spontaneous or assisted) can potentially impact on nephrogenesis and/or render the kidneys vulnerable to premature delivery and subsequent pathology. My findings in relation to the effects of IUGR on renal development and on renal function in the preterm neonate clearly support this concept. In addition to IUGR exerting a negative impact on the developing kidneys, there is the potential for many other intrauterine factors (some of which are common antecedents of preterm birth) to adversely impact renal development and renal function, especially in the event of preterm delivery.

7.4.1 INTRAUTERINE INFECTION AND INFLAMMATION (CHORIOAMNIONITIS)

Intrauterine infection (in particular, chorioamnionitis) is widely acknowledged as a major contributor to premature delivery (Mueller-Heubach et al., 1990, Goldenberg et al., 2000), especially in births prior to 32 weeks gestation (Mueller-Heubach et al., 1990, Lahra and Jeffery, 2004). A recent study by Ogge et al. (2011) found that chronic chorioamnionitis was a factor in 34% of deliveries relating to preterm labour with intact membranes, and 39% of cases of preterm labour with membrane rupture. Chorioamnionitis is defined as inflammation of the chorion and amnion, caused by a bacterial infection which typically ascends from the vagina

(Goldenberg et al., 2008). Importantly, chorioamnionitis can lead to fetal inflammatory response syndrome (FIRS) (Gantert et al., 2010), and this has been shown to adversely influence neonatal organ development. The effect of exposure to inflammation *in utero* on the fetal kidneys has recently been examined in a sheep model (Galinsky et al., 2011, Ryan et al., 2013). In the study by Galinsky et al. (2011), they observed a 20% reduction in nephron number, without any effect on body weight, when intrauterine inflammation was induced in late gestation using an acute intra-amniotic bolus dose of lipopolysaccharide (LPS, which initiates an inflammatory response similar to that observed with chorioamnionitis). Interestingly, however, when fetal lambs were exposed to a lower dose of LPS over a longer period (chronic exposure), at a time when nephrogenesis was rapidly ongoing, there were no observable detrimental effects on nephrogenesis (Ryan et al., 2013). The contrasting findings from these two studies demonstrate that the timing, duration and extent of infection/inflammation are important factors when assessing the impact of chorioamnionitis on the developing kidney.

7.4.2 MATERNAL DIABETES

Intrauterine exposure to maternal diabetes can significantly influence fetal growth throughout gestation and also induce preterm birth; this is of concern, given the recent rise in the incidence of Type 1 and Type 2 and/or gestational diabetes (Hunt and Schuller, 2007, Magon and Chauhan, 2012). A common consequence of intrauterine exposure to maternal diabetes is fetal macrosomia, in particular asymmetric macrosomia (Aerts et al., 1990). Macrosomia often leads to exaggerated fetal growth, whereby the baby is born with a birth weight that is high for gestational age (Jovanovic, 2001). This increase in body weight is a result of excessive amounts of glucose and other nutrients crossing the placenta. In contrast, when maternal diabetes (both Type 1 and Type 2) is severe, this can lead to IUGR in the infant (Rowan et al., 2009, Magon and Chauhan, 2012). With the increased prevalence of maternal diabetes there have been a

number of recent studies looking at the effects on the fetal kidneys. In a study conducted in preterm and term babies born to Pima Indian mothers, infants exposed to maternal diabetes (Type 2 diabetes) during gestation had higher levels of albumin excretion (3.8 times higher) when compared to infants of pre-diabetic and non-diabetic mothers, thus indicative of renal injury in offspring exposed to diabetes *in utero* (Nelson et al., 1998).

Animal studies have reported an increased incidence of renal malformations in offspring born to diabetic mothers (Type 1 diabetes) (Zhang et al., 2007, Tran et al., 2008). In particular, it has been shown that exposure to maternal diabetes can adversely impact nephrogenesis, with the offspring of diabetic mothers reported to have significantly smaller kidneys and glomerular size, accompanied by a 40% reduction in nephron endowment (Tran et al., 2008). Of concern, exposure to diabetes *in utero* led to greater glomerular and tubular apoptosis compared to offspring not exposed to diabetes, with the level of hyperglycaemia a strong determinant of the severity of the adverse effects observed in the kidneys (Tran et al., 2008). It is important to note that although many of the animal studies relate to the induction of Type 1 diabetes in the mothers, the findings in relation to fetal development are likely to be also relevant to maternal Type 2 diabetes and gestational diabetes, where the developing infant in all cases is exposed to hyperglycemia.

These previous studies create new avenues for future research. Indeed, it would be valuable to assess renin and nuclear factor-KappaB (NF-kB) expression in autopsied human IUGR fetal kidneys. It is suggested that maternal diabetes impairs nephrogenesis via enhanced intrarenal activation of the renin-angiotensin system and NF-kB signaling (Tran et al., 2008). Mice studies showed that NF-kB is an intracellular target in hyperglycaemia which is upregulated in kidneys of diabetic offspring (Mercurio and Manning, 1999). When combined with intrarenal renin-angiotensin system activation and a hyperglycemic environment *in utero*, studies have shown

upregulation of the NF-kB pathway leads to reduced kidney size with potential apoptosis of nephrons and a consequent nephron deficit (Mezzano et al., 2003, Lee et al., 2004). In mouse offspring, a change in renin expression together with increased angiotensinogen expression has the capability to stimulate increased angiotensin II formation, which could contribute to increased glomerular and tubular apoptosis, resulting in reduced kidney function.

7.4.3 ANTENATAL MEDICATIONS

In general, administration of medications during pregnancy is avoided wherever possible, due to potential adverse effects on the developing fetus. However, it is important to note that there are some medications that are specifically administered to women 'at risk' of delivering prematurely, and although these medications are considered safe, they do have the potential to adversely impact on the developing fetal kidneys.

7.4.3.1 GLUCOCORTICOIDS

When it is considered likely that a woman will deliver prematurely, she is routinely administered glucocorticoids, usually betamethasone. These medications have been shown to accelerate the maturation of the fetal lungs, and thus enhance the survival of the infant after preterm delivery (Shanks et al., 2010, Crowther et al., 2011). In addition to the effects in the newborn's lungs, the administration of glucocorticoids has also been observed to increase mean arterial blood pressure, renal blood flow and glomerular filtration rate (al-Dahan et al., 1987, Kari et al., 1994, van den Anker et al., 1994) indicating that the medications have a marked effect on renal function.

The effect of glucocorticoids on the developing kidney has been studied in a number of animal models, including the rat (Celsi et al., 1998, Ortiz et al., 2001, Ortiz et al., 2003), sheep (Stonestreet et al., 1983, Ervin et al., 1996, Wintour et al., 2003) and baboon (Ervin et al., 1998, Gubhaju et al., 2009). Findings suggest that exposure to glucocorticoids can adversely affect

nephron endowment and renal maturation. In sheep studies, administration of glucocorticoids during pregnancy (over 26 -28 days gestation) has been shown to significantly reduce nephron endowment in the exposed offspring (Moritz et al., 2011). In the neonatal rat, a reduction in glomerular density was observed when dexamethasone was administered at a time of ongoing postnatal nephrogenesis (de Vries et al., 2010). In our laboratory, we have looked at the effects of administration of antenatal glucocorticoids in a preterm baboon model (Gubhaju et al., 2009). Encouragingly, administration of antenatal glucocorticoids did not appear to have any adverse effects on the developing kidney, and nephron endowment was within the normal range (Gubhaju et al., 2009). However, there was a 9% increase in the number of developed (mature) glomeruli in the renal cortex in the betamethasone-exposed neonates, and a reduction in the width of the nephrogenic zone when compared to age-matched gestational controls. This suggests that there is accelerated renal maturation in response to glucocorticoid exposure, and this is in accordance with studies in other organs that have also shown accelerated maturation (Ervin et al., 1998, Jahnukainen et al., 2001).

7.4.3.2 ANTIBIOTICS

Infants that deliver preterm are often exposed to antibiotics *in utero*, as they are often prescribed to pregnant women with chorioamnionitis. Importantly, in this regard, antibiotics such as the aminoglycosides can readily cross the placenta (Pacifici, 2006) and there have been a number of experimental studies linking antibiotic exposure with an impairment of nephrogenesis (Gilbert et al., 1996, Rodriguez-Barbero et al., 1997, Giapros et al., 2003, Kent et al., 2007). For instance, it has been shown that the incubation of metanephroi with gentamicin in organ culture leads to decreased branching morphogenesis of the ureteric tree and thus reduced nephron formation (Gilbert et al., 1996). In addition, administration of

antibiotics to guinea pig and rat dams has been shown to lead to oligonephronia in the offspring (Gilbert et al., 1990).

7.4.3.3 INDOMETHACIN

Another medication routinely administered to women at risk of preterm birth is indomethacin. Indomethacin is a tocolytic drug, which functions to reduce prostaglandin synthesis; it is thereby highly effective at prolonging pregnancy (Kurki et al., 1991). Of concern, however, in rodent studies *in utero* exposure to indomethacin has been reported to reduce nephron endowment and reduce glomerular filtration rates (Pomeranz et al., 1996, Kent et al., 2009).

7.4.4 OLIGOHYDRAMNIOS

Oligohydramnios is characterised by reduced levels of amniotic fluid during pregnancy. It can manifest as a result of fetal renal injury, such as decreased renal blood flow and/or reduced renal perfusion, which ultimately leads to a reduction in fetal urine excretion, and consequently, the amount of amniotic fluid (Vanderheyden et al., 2003). Other causes that can contribute to a reduction in amniotic fluid include congenital anomalies such as: renal agenesis, polycystic kidneys, multicystic dysplastic kidneys and uretal or uretheral obstruction, or rupture of membranes (Nyberg et al., 2002). It has also been suggested that oligohydramnios can also result from bacterial infection within the amniotic cavity (such as chorioamnionitis), causing redistribution of blood flow within the developing fetus. A reduction in amniotic fluid at birth is often indicative of renal insufficiency in the neonate (Klaassen et al., 2007). The *In utero* detection of oligohydramnios often leads to the assisted induction of preterm labour, as oligohydramnios has been linked to a number of inauspicious pregnancy outcomes such as perinatal death, fetal distress labour, low birth weight and poor infant health at birth (Casey et al., 2000).

Collectively, there are many factors in the prenatal period that are linked to the onset of preterm birth that can potentially render the kidneys more vulnerable to impaired development and dysfunction postnatally, and these are all important areas for future research.

FINAL CONCLUSION

In conclusion, the clinically relevant findings of this thesis highlight the vulnerability of the kidneys to preterm birth, and this is exacerbated in Indigenous infants and/or those born IUGR. Given my findings, it is now important to develop strategies to monitor the long-term renal health of subjects born preterm and/or IUGR. This is particularly important in the Indigenous population, given that they have a high incidence of both preterm birth and IUGR. In future studies, it is also imperative to explore other antecedents of preterm birth as they may also confer an added renal vulnerability when birth comes early.

REFERENCES

AERTS, L., HOLEMANS, K. & VAN ASSCHE, F. A. 1990. Maternal diabetes during pregnancy: consequences for the offspring. *Diabetes Metab Rev,* 6**,** 147-67.

AIHW 2015. Australia's welfare 2015. Australia's welfare no. 12. Cat. no. AUS 189. Canberra: AIHW.

AL-DAHAN, J., STIMMLER, L., CHANTLER, C. & HAYCOCK, G. B. 1987. The effect of antenatal dexamethasone administration on glomerular filtration rate and renal sodium excretion in premature infants. *Pediatr Nephrol,* 1**,** 131-5.

BLACK, M. J., SUTHERLAND, M. R. & GUBHAJU, L. 2011. The effects of preterm birth on the kidney *In:* SAHAY, M. (ed.) *Basic Nephrology and Acute Kidney Injury.* Rijeka Croatia: InTech.

BOLIGNANO, D., LACQUANITI, A., COPPOLINO, G., DONATO, V., CAMPO, S., FAZIO, M. R., NICOCIA, G. & BUEMI, M. 2009. Neutrophil gelatinase-associated lipocalin (NGAL) and progression of chronic kidney disease. *Clin J Am Soc Nephrol,* 4**,** 337-44.

CAIN, J. E., DI GIOVANNI, V., SMEETON, J. & ROSENBLUM, N. D. 2010. Genetics of renal hypoplasia: insights into the mechanisms controlling nephron endowment. *Pediatr Res,* 68**,** 91-8.

CASEY, B. M., MCINTIRE, D. D., BLOOM, S. L., LUCAS, M. J., SANTOS, R., TWICKLER, D. M., RAMUS, R. M. & LEVENO, K. J. 2000. Pregnancy outcomes after antepartum diagnosis of oligohydramnios at or beyond 34 weeks' gestation. *Am J Obstet Gynecol,* 182**,** 909-12.

CEBRIAN, C., ASAI, N., D'AGATI, V. & COSTANTINI, F. 2014. The number of fetal nephron progenitor cells limits ureteric branching and adult nephron endowment. *Cell Rep,* 7**,** 127-37.

CELSI, G., KISTNER, A., AIZMAN, R., EKLOF, A. C., CECCATELLI, S., DE SANTIAGO, A. & JACOBSON, S. H. 1998. Prenatal dexamethasone causes oligonephronia, sodium retention, and higher blood pressure in the offspring. *Pediatr Res,* 44**,** 317-22.

CONTI, M., MOUTEREAU, S., ZATER, M., LALLALI, K., DURRBACH, A., MANIVET, P., ESCHWEGE, P. & LORIC, S. 2006. Urinary cystatin C as a specific marker of tubular dysfunction. *Clin Chem Lab Med,* 44**,** 288-91.

CROWTHER, C. A., MCKINLAY, C. J., MIDDLETON, P. & HARDING, J. E. 2011. Repeat doses of prenatal corticosteroids for women at risk of preterm birth for improving neonatal health outcomes. *Cochrane Database Syst Rev***,** CD003935.

CULLEN-MCEWEN, L., SUTHERLAND, M. R. & BLACK, M. J. 2016. Chapter 3 - The Human Kidney: Parallels in Structure, Spatial Development, and Timing of Nephrogenesis A2 - Little, Melissa H. *Kidney Development, Disease, Repair and Regeneration.* San Diego: Academic Press.

CULLEN-MCEWEN, L. A., DRAGO, J. & BERTRAM, J. F. 2001. Nephron endowment in glial cell line-derived neurotrophic factor (GDNF) heterozygous mice. *Kidney International,* 60**,** 31-36.

DE VRIES, W. B., VAN DEN BORNE, P., GOLDSCHMEDING, R., DE WEGER, R. A., BAL, M. P., VAN BEL, F. & VAN OOSTERHOUT, M. F. 2010. Neonatal dexamethasone treatment in the rat leads to kidney damage in adulthood. *Pediatr Res,* 67**,** 72-6.

DOREY, E. S., PANTALEON, M., WEIR, K. A. & MORITZ, K. M. 2014. Adverse prenatal environment and kidney development: implications for programing of adult disease. *Reproduction,* 147**,** R189-98.

DU CHEYRON, D., FRADIN, S., RAMAKERS, M., TERZI, N., GUILLOTIN, D., BOUCHET, B., DAUBIN, C. & CHARBONNEAU, P. 2008. Angiotensin converting enzyme insertion/deletion genetic polymorphism: its impact on renal function in critically ill patients. *Crit Care Med,* 36**,** 3178-83.

DUFFY, D. L., MCDONALD, S. P., HAYHURST, B., PANAGIOTOPOULOS, S., SMITH, T. J., WANG, X. L., WILCKEN, D. E., DUARTE, N. L., MATHEWS, J. & HOY, W. E. 2016. Familial aggregation of albuminuria and arterial hypertension in an Aboriginal Australian community and the contribution of variants in ACE and TP53. *BMC Nephrol,* 17**,** 183.

EMAMIAN, S. A., NIELSEN, M. B., PEDERSEN, J. F. & YTTE, L. 1993. Kidney dimensions at sonography: correlation with age, sex, and habitus in 665 adult volunteers. *AJR Am J Roentgenol,* 160**,** 83-6.

ERVIN, M. G., BERRY, L. M., IKEGAMI, M., JOBE, A. H., PADBURY, J. F. & POLK, D. H. 1996. Single dose fetal betamethasone administration stabilizes postnatal glomerular filtration rate and alters endocrine function in premature lambs. *Pediatr Res,* 40**,** 645-51.

ERVIN, M. G., SEIDNER, S. R., LELAND, M. M., IKEGAMI, M. & JOBE, A. H. 1998. Direct fetal glucocorticoid treatment alters postnatal adaptation in premature newborn baboons. *Am J Physiol,* 274**,** R1169-76.

FONSECA FERRAZ, M. L., DOS SANTOS, A. M., CAVELLANI, C. L., ROSSI, R. C., CORREA, R. R., DOS REIS, M. A., DE PAULA ANTUNES TEIXEIRA, V. & DA CUNHA CASTRO, E. C. 2008. Histochemical and immunohistochemical study of the glomerular development in human fetuses. *Pediatr Nephrol,* 23**,** 257-62.

GALINSKY, R., MOSS, T. J., GUBHAJU, L., HOOPER, S. B., BLACK, M. J. & POLGLASE, G. R. 2011. Effect of intra-amniotic lipopolysaccharide on nephron number in preterm fetal sheep. *Am J Physiol Renal Physiol,* 301**,** F280-5.

GANTERT, M., BEEN, J. V., GAVILANES, A. W., GARNIER, Y., ZIMMERMANN, L. J. & KRAMER, B. W. 2010. Chorioamnionitis: a multiorgan disease of the fetus? *J Perinatol,* 30 Suppl**,** S21-30.

GIAPROS, V. I., ANDRONIKOU, S. K., CHOLEVAS, V. I. & PAPADOPOULOU, Z. L. 2003. Renal function and effect of aminoglycoside therapy during the first ten days of life. *Pediatr Nephrol,* 18**,** 46-52.

GILBERT, T., CIBERT, C., MOREAU, E., GERAUD, G. & MERLET-BENICHOU, C. 1996. Early defect in branching morphogenesis of the ureteric bud in induced nephron deficit. *Kidney Int,* 50**,** 783-95.

GILBERT, T., LELIEVRE-PEGORIER, M. & MERLET-BENICHOU, C. 1990. Immediate and long-term renal effects of fetal exposure to gentamicin. *Pediatr Nephrol,* 4**,** 445-50.

GOLDENBERG, R. L., CULHANE, J. F., IAMS, J. D. & ROMERO, R. 2008. Epidemiology and causes of preterm birth. *Lancet,* 371**,** 75-84.

GOLDENBERG, R. L., HAUTH, J. C. & ANDREWS, W. W. 2000. Intrauterine infection and preterm delivery. *N Engl J Med,* 342**,** 1500-7.

GUBHAJU, L., SUTHERLAND, M. R., YODER, B. A., ZULLI, A., BERTRAM, J. F. & BLACK, M. J. 2009. Is nephrogenesis affected by preterm birth? Studies in a non-human primate model. *Am J Physiol Renal Physiol,* 297**,** F1668-77.

HINCHLIFFE, S. A., LYNCH, M. R., SARGENT, P. H., HOWARD, C. V. & VAN VELZEN, D. 1992. The effect of intrauterine growth retardation on the development of renal nephrons. *Br J Obstet Gynaecol,* 99**,** 296-301.

HINCHLIFFE, S. A., SARGENT, P. H., HOWARD, C. V., CHAN, Y. F. & VAN VELZEN, D. 1991. Human intrauterine renal growth expressed in absolute number of glomeruli assessed by the disector method and Cavalieri principle. *Lab Invest,* 64**,** 777-84.

HOPPE, C. C., EVANS, R. G., MORITZ, K. M., CULLEN-MCEWEN, L. A., FITZGERALD, S. M., DOWLING, J. & BERTRAM, J. F. 2007. Combined prenatal and postnatal protein restriction influences adult kidney structure, function, and arterial pressure. *Am J Physiol Regul Integr Comp Physiol,* 292**,** R462-9.

HOY, W. E., HUGHSON, M. D., BERTRAM, J. F., DOUGLAS-DENTON, R. & AMANN, K. 2005. Nephron number, hypertension, renal disease, and renal failure. *J Am Soc Nephrol,* 16**,** 2557-64.

HOY, W. E., HUGHSON, M. D., SINGH, G. R., DOUGLAS-DENTON, R. & BERTRAM, J. F. 2006. Reduced nephron number and glomerulomegaly in Australian Aborigines: a group at high risk for renal disease and hypertension. *Kidney Int,* 70**,** 104-10.

HOY, W. E., REES, M., KILE, E., MATHEWS, J. D., MCCREDIE, D. A., PUGSLEY, D. J. & WANG, Z. 1998. Low birthweight and renal disease in Australian aborigines. *The Lancet,* 352**,** 1826-1827.

HOY, W. E., REES, M., KILE, E., MATHEWS, J. D. & WANG, Z. 1999. A new dimension to the Barker hypothesis: Low birth weight and susceptibility to renal disease. *Kidney International,* 56**,** 1072-1077.

HOY, W. E., SAMUEL, T., MOTT, S. A., KINCAID-SMITH, P. S., FOGO, A. B., DOWLING, J. P., HUGHSON, M. D., SINNIAH, R., PUGSLEY, D. J., KIRUBAKARAN, M. G., DOUGLAS-DENTON, R. N. & BERTRAM, J. F. 2012. Renal biopsy findings among Indigenous Australians: a nationwide review. *Kidney Int,* 82**,** 1321-31.

HUNT, K. J. & SCHULLER, K. L. 2007. The increasing prevalence of diabetes in pregnancy. *Obstet Gynecol Clin North Am,* 34**,** 173-99, vii.

INGELFINGER, J. R. 2008. Disparities in renal endowment: causes and consequences. *Adv Chronic Kidney Dis,* 15**,** 107-14.

INGEMARSSON, I. 2003. Gender aspects of preterm birth. *BJOG,* 110 Suppl 20**,** 34-8.

JAHNUKAINEN, T., CHEN, M., BERG, U. & CELSI, G. 2001. Antenatal glucocorticoids and renal function after birth. *Semin Neonatol,* 6**,** 351-5.

JOVANOVIC, L. 2001. What is so bad about a big baby? *Diabetes Care,* 24**,** 1317-8.

KACZMARCZYK, M., LONIEWSKA, B., KUPRJANOWICZ, A., JOZWA, A., BINCZAK-KULETA, A., GORACY, I., DAWID, G., KORDEK, A., KARPINSKA-KACZMARCZYK, K., BRODKIEWICZ, A. & CIECHANOWICZ, A. 2013. An insertion/deletion ACE polymorphism and kidney size in Polish full-term newborns. *J Renin Angiotensin Aldosterone Syst,* 14**,** 369-74.

KARI, M., HALLMA, M., ERONEN, M., TERAMO, K., VIRTANEN, M., KOIVISTO, M. & IKONEN, R. 1994. Prenatal dexamethasone treatment in conjunction with rescue therapy of human surfactant: a randomized placebo-controlled multicenter study. *Pediatrics,* 93**,** 730-6.

KEIJZER-VEEN, M., FINKEN, M., NAUTA, J., DEKKER, F., HILLE, E., FROLICH, M., WIT, J. & VAN DER HEIJDEN, A. 2005 Is blood pressure increased 19 years after intrauterine growth restriction and preterm birth? A prospective follow-up study in The Netherlands. *Pediatrics,* 116**,** 725-31.

KENT, A. L., DOUGLAS-DENTON, R., SHADBOLT, B., DAHLSTROM, J. E., MAXWELL, L. E., KOINA, M. E., FALK, M. C., WILLENBORG, D. & BERTRAM, J. F. 2009. Indomethacin, ibuprofen and gentamicin administered during late stages of glomerulogenesis do not reduce glomerular number at 14 days of age in the neonatal rat. *Pediatric Nephrology,* 24**,** 1143-9.

KENT, A. L., MAXWELL, L. E., KOINA, M. E., FALK, M. C., WILLENBORG, D. & DAHLSTROM, J. E. 2007. Renal glomeruli and tubular injury following indomethacin, ibuprofen, and gentamicin exposure in a neonatal rat model. *Pediatr Res,* 62**,** 307-12.

KENT, A. L., WRIGHT, I. M., ABDEL-LATIF, M. E., NEW SOUTH, W. & AUSTRALIAN CAPITAL TERRITORY NEONATAL INTENSIVE CARE UNITS AUDIT, G. 2012. Mortality and adverse neurologic outcomes are greater in preterm male infants. *Pediatrics,* 129**,** 124-31.

KLAASSEN, I., NEUHAUS, T. J., MUELLER-WIEFEL, D. E. & KEMPER, M. J. 2007. Antenatal oligohydramnios of renal origin: long-term outcome. *Nephrol Dial Transplant,* 22**,** 432-9.

KONJE, J. C., BELL, S. C., MORTON, J. J., DE CHAZAL, R. & TAYLOR, D. J. 1996. Human fetal kidney morphometry during gestation and the relationship between weight, kidney morphometry and plasma active renin concentration at birth. *Clin Sci,* 91**,** 169-75.

KURKI, T., ERONEN, M., LUMME, R. & YLIKORKALA, O. 1991. A randomized double-dummy comparison between indomethacin and nylidrin in threatened preterm labor. *Obstet Gynecol,* 78**,** 1093-7.

LAHRA, M. M. & JEFFERY, H. E. 2004. A fetal response to chorioamnionitis is associated with early survival after preterm birth. *Am J Obstet Gynecol,* 190**,** 147-151.

LANGLEY-EVANS, S. C., WELHAM, S. J. & JACKSON, A. A. 1999. Fetal exposure to a maternal low protein diet impairs nephrogenesis and promotes hypertension in the rat. *Life Sci,* 64**,** 965-74.

LASAGNI, L. & ROMAGNANI, P. 2010. Glomerular epithelial stem cells: the good, the bad, and the ugly. *J Am Soc Nephrol,* 21**,** 1612-9.

LAWN, J. E. & KINNEY, M. 2014. Preterm birth: now the leading cause of child death worldwide. *Sci Transl Med,* 6**,** 263ed21.

LAZZERI, E., MAZZINGHI, B. & ROMAGNANI, P. 2010. Regeneration and the kidney. *Curr Opin Nephrol Hypertens,* 19**,** 248-53.

LEE, F. T., CAO, Z., LONG, D. M., PANAGIOTOPOULOS, S., JERUMS, G., COOPER, M. E. & FORBES, J. M. 2004. Interactions between angiotensin II and NF-kappaB-dependent pathways in modulating macrophage infiltration in experimental diabetic nephropathy. *J Am Soc Nephrol,* 15**,** 2139-51.

LI, Z., ZEKI, R., HILDER, L. & SULLIVAN, E. 2012. Australia's mothers and babies 2010. Perinatal statistics series no. 27.

Cat. no. PER 57. Canberra. *AIHW National Perinatal Epidemiology and Statistics Unit*.

MAGON, N. & CHAUHAN, M. 2012. Pregnancy in Type 1 Diabetes Mellitus: How Special are Special Issues? *N Am J Med Sci,* 4**,** 250-6.

MAJUMDAR, A., VAINIO, S., KISPERT, A., MCMAHON, J. & MCMAHON, A. P. 2003. Wnt11 and Ret/Gdnf pathways cooperate in regulating ureteric branching during metanephric kidney development. *Development,* 130**,** 3175-85.

MANALICH, R., REYES, L., HERRERA, M., MELENDI, C. & FUNDORA, I. 2000. Relationship between weight at birth and the number and size of renal glomeruli in humans: a histomorphometric study. *Kidney Int,* 58**,** 770-3.

MERCURIO, F. & MANNING, A. M. 1999. NF-kappaB as a primary regulator of the stress response. *Oncogene,* 18**,** 6163-71.

MEZZANO, S., DROGUETT, A., BURGOS, M. E., ARDILES, L. G., FLORES, C. A., AROS, C. A., CAORSI, I., VIO, C. P., RUIZ-ORTEGA, M. & EGIDO, J. 2003. Renin-angiotensin system activation and interstitial inflammation in human diabetic nephropathy. *Kidney Int Suppl***,** S64-70.

MITCHELL, E. K., LOUEY, S., COCK, M. L., HARDING, R. & BLACK, M. J. 2004. Nephron endowment and filtration surface area in the kidney after growth restriction of fetal sheep. *Pediatr Res,* 55**,** 769-73.

MORITZ, K. M. & CULLEN-MCEWEN, L. A. 2006. Kidney development and fetal programming. *In:* WINTOUR, E. M. & OWENS, J. A. (eds.) *Early Life Origins of Health and Disease.* New York: Eurekah.com & Springer Science+Business Media.

MORITZ, K. M., DE MATTEO, R., DODIC, M., JEFFERIES, A. J., ARENA, D., WINTOUR, E. M., PROBYN, M. E., BERTRAM, J. F., SINGH, R. R., ZANINI, S. & EVANS, R. G. 2011. Prenatal glucocorticoid exposure in the sheep alters renal development in utero: implications for adult renal function and blood pressure control. *Am J Physiol Regul Integr Comp Physiol,* 301**,** R500-9.

MORITZ, K. M., MAZZUCA, M. Q., SIEBEL, A. L., MIBUS, A., ARENA, D., TARE, M., OWENS, J. A. & WLODEK, M. E. 2009. Uteroplacental insufficiency causes a nephron deficit, modest renal insufficiency but no hypertension with ageing in female rats. *J Physiol,* 587**,** 2635-46.

MUELLER-HEUBACH, E., RUBINSTEIN, D. N. & SCHWARZ, S. S. 1990. Histologic chorioamnionitis and preterm delivery in different patient populations. *Obstet Gynecol,* 75**,** 622-6.

NADASDY, T., LASZIK, Z., BLICK, K. E., JOHNSON, L. D. & SILVA, F. G. 1994. Proliferative activity of intrinsic cell populations in the normal human kidney. *J Am Soc Nephrol,* 4**,** 2032-9.

NELSON, R. G., MORGENSTERN, H. & BENNETT, P. H. 1998. Intrauterine diabetes exposure and the risk of renal disease in diabetic Pima Indians. *Diabetes,* 47**,** 1489-93.

NYBERG, D. A., MCGAHAN, J. P., PRETORIUS, D. H. & PILU, G. 2002. Diagnostic imaging of fetal anomalies. *Lippincott Williams &Wilkins, Philadelphia*.

OGGE, G., ROMERO, R., LEE, D. C., GOTSCH, F., THAN, N. G., LEE, J., CHAIWORAPONGSA, T., DONG, Z., MITTAL, P., HASSAN, S. S. & KIM, C. J. 2011. Chronic chorioamnionitis displays distinct alterations of the amniotic fluid proteome. *J Pathol,* 223**,** 553-65.

ORTIZ, L. A., QUAN, A., WEINBERG, A. & BAUM, M. 2001. Effect of prenatal dexamethasone on rat renal development. *Kidney International,* 59**,** 1663-9.

ORTIZ, L. A., QUAN, A., ZARZAR, F., WEINBERG, A. & BAUM, M. 2003. Prenatal dexamethasone programs hypertension and renal injury in the rat. *Hypertension,* 41**,** 328-34.

OSATHANONDH, V. & POTTER, E. L. 1963. Development of Human Kidney as Shown by Microdissection. Iii. Formation and Interrelationship of Collecting Tubules and Nephrons. *Arch Pathol,* 76**,** 290-302.

PACIFICI, G. M. 2006. Placental transfer of antibiotics administered to the mother: a review. *Int J Clin Pharmacol Ther,* 44**,** 57-63.

PEACOCK, P. J., HENDERSON, J., ODD, D. & EMOND, A. 2012. Early school attainment in late-preterm infants. *Arch Dis Child,* 97**,** 118-20.

POMERANZ, A., KORZETS, Z., DOLFIN, Z., ELIAKIM, A., BERNHEIM, J. & WOLACH, B. 1996. Acute renal failure in the neonate induced by the administration of indomethacin as a tocolytic agent. *Nephrol Dial Transplant,* 11**,** 1139-41.

PUELLES, V. G., DOUGLAS-DENTON, R. N., CULLEN-MCEWEN, L. A., LI, J., HUGHSON, M. D., HOY, W. E., KERR, P. G. & BERTRAM, J. F. 2015. Podocyte Number in Children and Adults: Associations with Glomerular Size and Numbers of Other Glomerular Resident Cells. *J Am Soc Nephrol,* 26**,** 2277-88.

QUINLAN, J., LEMIRE, M., HUDSON, T., QU, H., BENJAMIN, A., ROY, A., PASCUET, E., GOODYER, M., RAJU, C., ZHANG, Z., HOUGHTON, F. & GOODYER, P. 2007. A common variant of the PAX2 gene is associated with reduced newborn kidney size. *J Am Soc Nephrol,* 18**,** 1915-21.

RAKOW, A., JOHANSSON, S., LEGNEVALL, L., SEVASTIK, R., CELSI, G., NORMAN, M. & VANPEE, M. 2008. Renal volume and function in school-age children born preterm or small for gestational age. *Pediatr Nephrol,* 23**,** 1309-15.

RODRIGUEZ-BARBERO, A., LOPEZ-NOVOA, J. M. & AREVALO, M. 1997. Involvement of platelet-activating factor in gentamicin nephrotoxicity in rats. *Exp Nephrol,* 5**,** 47-54.

ROWAN, J. A., LUEN, S., HUGHES, R. C., SADLER, L. C. & MCCOWAN, L. M. 2009. Customised birthweight centiles are useful for identifying small-for-gestational-age babies in women with type 2 diabetes. *Aust N Z J Obstet Gynaecol,* 49**,** 180-4.

RYAN, D., ATIK, A., DE MATTEO, R., HARDING, R. & BLACK, M. J. 2013. Chronic intrauterine exposure to endotoxin does not alter fetal nephron number or glomerular size. *Clin Exp Pharmacol Physiol*.

SAHAJPAL, V. & ASHTON, N. 2005. Increased glomerular angiotensin II binding in rats exposed to a maternal low protein diet in utero. *J Physiol,* 563**,** 193-201.

SALOMON, R., TELLIER, A. L., ATTIE-BITACH, T., AMIEL, J., VEKEMANS, M., LYONNET, S., DUREAU, P., NIAUDET, P., GUBLER, M. C. & BROYER, M. 2001. PAX2 mutations in oligomeganephronia. *Kidney Int,* 59**,** 457-62.

SATYAPAL, K. S., KALIDEEN, J. M., SINGH, B., HAFFEJEE, A. A. & ROBBS, J. V. 2003. Why we use the donor left kidney in live related transplantation. *S Afr J Surg,* 41**,** 24-6.

SHAKYA, R., WATANABE, T. & COSTANTINI, F. 2005. The role of GDNF/Ret signaling in ureteric bud cell fate and branching morphogenesis. *Dev Cell,* 8**,** 65-74.

SHANKLAND, S. J., ANDERS, H. J. & ROMAGNANI, P. 2013. Glomerular parietal epithelial cells in kidney physiology, pathology, and repair. *Curr Opin Nephrol Hypertens,* 22**,** 302-9.

SHANKS, A., GROSS, G., SHIM, T., ALLSWORTH, J., SADOVSKY, Y. & BILDIRICI, I. 2010. Administration of steroids after 34 weeks of gestation enhances fetal lung maturity profiles. *Am J Obstet Gynecol*.

SILVER, L. E., DECAMPS, P. J., KORST, L. M., PLATT, L. D. & CASTRO, L. C. 2003. Intrauterine growth restriction is accompanied by decreased renal volume in the human fetus. *American Journal of Obstetrics and Gynecology,* 188**,** 1320-1325.

SINGH, G. R. & HOY, W. E. 2004. Kidney volume, blood pressure, and albuminuria: findings in an Australian aboriginal community. *Am J Kidney Dis,* 43**,** 254-9.

SOUSTER, L. P. & EMERY, J. L. 1980. The sizes of renal glomeruli in fetuses and infants. *J Anat,* 130**,** 595-602.

STONESTREET, B. S., HANSEN, N. B., LAPTOOK, A. R. & OH, W. 1983. Glucocorticoid accelerates renal functional maturation in fetal lambs. *Early Hum Dev,* 8**,** 331-41.

STUMPERS, S. & THOMSON, N. 2013. Review of kidney disease and urological disorders among indigenous people. *Australian Indigenous HealthInfoNet*.

TRAN, S., CHEN, Y. W., CHENIER, I., CHAN, J. S., QUAGGIN, S., HEBERT, M. J., INGELFINGER, J. R. & ZHANG, S. L. 2008. Maternal diabetes modulates renal morphogenesis in offspring. *Journal of the American Society of Nephrology,* 19**,** 943-52.

TREUTING, P. M. & KOWALEWSKA, J. 2011. Urinary System. *Comparative Anatomy and Histology: A Mouse and Human Atlas***,** 229-251.

TUNDIDOR, D., GARCIA-PATTERSON, A., MARIA, M. A., UBEDA, J., GINOVART, G., ADELANTADO, J. M., DE LEIVA, A. & CORCOY, R. 2012. Perinatal maternal and neonatal outcomes in women with gestational diabetes mellitus according to fetal sex. *Gend Med,* 9**,** 411-7.

VAIDYA, V. S., RAMIREZ, V., ICHIMURA, T., BOBADILLA, N. A. & BONVENTRE, J. V. 2006. Urinary kidney injury molecule-1: a sensitive quantitative biomarker for early detection of kidney tubular injury. *Am J Physiol Renal Physiol,* 290**,** F517-29.

VAN DEN ANKER, J. N., HOP, W. C., DE GROOT, R., VAN DER HEIJDEN, B. J., BROERSE, H. M., LINDEMANS, J. & SAUER, P. J. 1994. Effects of prenatal exposure to betamethasone and indomethacin on the glomerular filtration rate in the preterm infant. *Pediatr Res,* 36**,** 578-81.

VANDERHEYDEN, T., KUMAR, S. & FISK, N. M. 2003. Fetal renal impairment. *Semin Neonatol,* 8**,** 279-89.

WHITE, S. L., PERKOVIC, V., CASS, A., CHANG, C. L., POULTER, N. R., SPECTOR, T., HAYSOM, L., CRAIG, J. C., SALMI, I. A., CHADBAN, S. J. & HUXLEY, R. R. 2009. Is low birth weight an antecedent of CKD in later life? A systematic review of observational studies. *Am J Kidney Dis,* 54**,** 248-61.

WINTOUR, E. M., MORITZ, K. M., JOHNSON, K., RICARDO, S., SAMUEL, C. S. & DODIC, M. 2003. Reduced nephron number in adult sheep, hypertensive as a result of prenatal glucocorticoid treatment. *J Physiol,* 549**,** 929-35.

WLODEK, M. E., WESTCOTT, K., SIEBEL, A. L., OWENS, J. A. & MORITZ, K. M. 2008. Growth restriction before or after birth reduces nephron number and increases blood pressure in male rats. *Kidney Int,* 74**,** 187-95.

WOODS, L., WEEKS, D. & RASCH, R. 2004. Programming of adult blood pressure by maternal protein restriction: role of nephrogenesis. *Kidney International,* 65**,** 1339-48.

WOOLF, A. S., WINYARD, P. J., HERMANNS, M. H. & WELHAM, S. M. J. 2003. Maldevelopment of the Human Kidney and Lower Urinary Tract: An Overview. *In:* VIZE PD, W. A., BARD JBL, (ed.) *The Kidney: From Normal Development to Congenital Disease.* Academic Press.

YANG, B., EL NAHAS, A. M., THOMAS, G. L., HAYLOR, J. L., WATSON, P. F., WAGNER, B. & JOHNSON, T. S. 2001. Caspase-3 and apoptosis in experimental chronic renal scarring. *Kidney Int,* 60**,** 1765-76.

ZERIN, J. M. & MEYER, R. D. 2000. Sonographic assessment of renal length in the first year of life: the problem of "spurious nephromegaly". *Pediatr Radiol,* 30**,** 52-7.

ZHANG, S. L., CHEN, Y. W., TRAN, S., CHENIER, I., HEBERT, M. J. & INGELFINGER, J. R. 2007. Reactive oxygen species in the presence of high glucose alter ureteric bud morphogenesis. *J Am Soc Nephrol,* 18**,** 2105-15.

ZHANG, Z., QUINLAN, J., HOY, W., HUGHSON, M. D., LEMIRE, M., HUDSON, T., HUEBER, P. A., BENJAMIN, A., ROY, A., PASCUET, E., GOODYER, M., RAJU, C., HOUGHTON, F., BERTRAM, J. & GOODYER, P. 2008. A common RET variant is associated with reduced newborn kidney size and function. *J Am Soc Nephrol,* 19**,** 2027-34.

ZIMANYI, M., BERTRAM, J. F. & BLACK, M. J. 2004. Does a Nephron Deficit in Rats Predispose to Salt-Sensitive Hypertension? *Kidney and Blood Pressure Research,* 27**,** 239-247.

ZIMANYI, M. A., DENTON, K. M., FORBES, J. M., THALLAS-BONKE, V., THOMAS, M. C., POON, F. & BLACK, M. J. 2006. A developmental nephron deficit in rats is associated with increased susceptibility to a secondary renal injury due to advanced glycation end-products. *Diabetologia,* 49**,** 801-10.

ZOHDI, V., MORITZ, K. M., BUBB, K. J., COCK, M. L., WREFORD, N., HARDING, R. & BLACK, M. J. 2007. Nephrogenesis and the renal renin-angiotensin system in fetal sheep: effects of intrauterine growth restriction during late gestation. *Am J Physiol Regul Integr Comp Physiol,* 293**,** R1267-73.

ZOHDI, V., SUTHERLAND, M. R., LIM, K., GUBHAJU, L., ZIMANYI, M. A. & BLACK, M. J. 2012. Low Birth Weight due to Intrauterine Growth Restriction and/or Preterm Birth: Effects on Nephron Number and Long-Term Renal Health. *Int J Nephrol,* 2012**,** 136942.