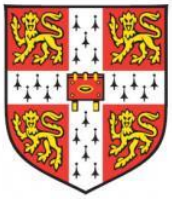
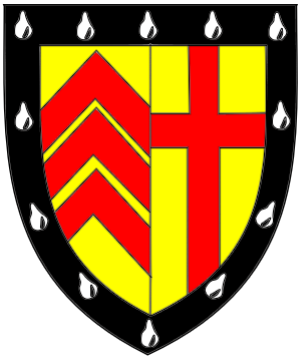


UNIVERSITY OF CAMBRIDGE

Exploring the impact of maternal obesity on offspring renal morphology and later life health



Clare College

Adele Grace Pinnock

October 2017

This dissertation is submitted for the degree of Doctor of Philosophy

Exploring the impact of maternal obesity on offspring renal morphology and later life health

Adele Pinnock

Summary

It is well established that exposure to adverse environments in early life including both maternal under and over-nutrition predisposes individuals to similar adverse traditionally adult onset diseases such as the metabolic syndrome. Epidemiological observations and animal models have highlighted that early life exposure to maternal under-nutrition has a detrimental effect on offspring kidney health. The prevalence of chronic kidney disease has increased rapidly in recent years, concurrently with the growing obesity epidemic. Obesity is now prevalent in all age groups within the population including women of child-bearing age. Despite this, the effect of early life exposure to maternal obesity on long-term kidney health has not been investigated in humans.

Studies in animals have demonstrated that exposure to early life under-nutrition programs the offspring kidney. Offspring exposed to maternal calorie restriction or a low protein diet typically display a reduced number of nephrons and increased glomerular areas. No studies to date have investigated the effect of maternal obesity on early life kidney and glomerular morphology. To address this, as part of this thesis, kidney morphology was assessed at weaning in male mice exposed to maternal diet-induced obesity throughout gestation and lactation. There was no effect of maternal diet on the number of nephrons counted within a distinct region in the offspring kidneys. However, glomerular density was decreased and glomerular area was increased in offspring exposed to maternal obesity.

Alterations in renal morphology in early life have been linked to hypertension and renal disease in adulthood in both epidemiological and animal studies. Therefore, a second aim of this thesis was to assess blood pressure, renal function and markers of renal damage in offspring exposed to maternal obesity throughout the life-course. Post-pubescent male offspring (8 weeks of age) exposed to maternal obesity displayed increased blood pressure but no signs of renal dysfunction or damage. However, by six months of age offspring exposed to maternal obesity had increased glomerulosclerosis and tubulointerstitial fibrosis.

The obesity epidemic is attributed to a shift in behaviours towards consumption of energy dense foods and inactivity. In addition, evidence from human and animal studies has highlighted that exposure to maternal obesity primes offspring to prefer sugary and fatty foods and to consume more calories. As

such, offspring exposed to maternal obesity are likely to encounter an obesogenic environment in later life. A third aim of this thesis was therefore to determine the effect of maternal obesity in combination with a post-weaning obesogenic diet on offspring kidney health. To address this aim, offspring either exposed to an obesogenic diet or control diet throughout pregnancy and lactation were weaned onto either an obesogenic or control diet themselves. Six month old offspring exposed to a post-weaning obesity alone displayed indices of renal dysfunction and damage including glomerulosclerosis and tubulointerstitial fibrosis. Importantly, exposure to maternal obesity exacerbated the renal fibrosis in offspring exposed to a post-weaning obesogenic diet.

With the growing prevalence of maternal obesity globally, there is great interest in determining an effective intervention to prevent adverse health outcomes in exposed individuals. The Ozanne laboratory has shown that maternal exercise in obese dams during pregnancy reduces maternal serum insulin and offspring insulin to control levels, highlighting that maternal exercise may be a promising intervention to limit adult-onset diseases in offspring exposed to early life obesity. The final aim of this thesis was to therefore assess the effect of exercise during an obese pregnancy on markers of offspring renal development during late gestation. Gene markers of ureteric bud branching, an important precursor of nephrogenesis, were increased in fetuses exposed to maternal obesity with exercise as opposed to obesity alone. Additionally one of these gene markers correlated negatively with maternal insulin levels. Protein markers indicative of an active ureteric bud branching pathway were also increased in offspring exposed to maternal obesity with exercise.

In conclusion, studies conducted in this thesis demonstrate that offspring exposed to maternal obesity show alterations in renal morphology in early life and are predisposed for renal disease in later life, especially when they are challenged with a post-weaning obesogenic diet. Maternal exercise might be an effective intervention to rescue offspring renal morphology and later life health associated with maternal obesity, however this requires further investigation. These results have important implications for future generations within the setting of an ever increasing obesity epidemic and a growing prevalence of chronic kidney diseases.

Declaration

The research described in this thesis was conducted between October 2014 and September 2017 in the Department of Clinical Biochemistry at the University of Cambridge. This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration except as specified in the text. This thesis has not been submitted for any other degree, diploma or other qualification and excluding figures, tables, appendices and bibliography does not exceed 60,000 words.

Adele Pinnock

October 2017

Publications

Pinnock AG and Ozanne SE, 2016. Early Epigenetic Origins of Adult Disease. eLS. 1–7.

Loche E, Blackmore HL, Carpenter AA, Beeson J, **Pinnock AG**, Ashmore TJ, Aiken CE, Almeida Faria J, Schoonejans J, Giussani DA, Fernandez-Twinn D, Ozanne SE, 2018. Maternal diet-induced obesity programs cardiac dysfunction in male mice independently of post-weaning diet. Cardiovascular Research. doi: 10.1093/cvr/cvy082.

Pinnock AG, Ashmore T, Blackmore H, Pantaleao L & Ozanne SE. Early life exposure to maternal obesity promotes poor renal health in male mice. (in preparation)

Abstracts

2014 IMS student symposium, Cambridge, UK – Poster prize

2015 Exercise conference, Cambridge, UK – Poster

2016 BSCR/BAS Spring meeting, Manchester, UK – Poster

2016 IMS student symposium – Talk prize

2017 Annual BHF Student Meeting, London, UK – Poster

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Abbreviations list

°C – Degrees Celsius

µm – micrometres

μm^2 – micrometres squared (area)

µl – microlitres

µmol – micromoles

11β-HSD - 11β-Hydroxysteroid Dehydrogenase

ACE – Angiotensin converting enzyme

ACTH - adrenocorticotropic hormone

AGA – Appropriate for gestational age

Agtr1 – Angiotensin II receptor type 1 gene

Agtr2 - Angiotensin II receptor type 1 gene

ANOVA – Analysis of Variance

APS - Ammonium Persulfate

ATR1 - angiotensin II receptor type 1

ATR2 - angiotensin II receptor type 2

AUC – Area Under the Curve

AVP – Arginine Vasopressin

Bax - BCL2 associated X, Apoptosis regulator gene

BMI – Body Mass Index

Bmp4 - bone morphogenetic protein 4 gene

BSA – Bovine Serum Albumin

BUN – Blood Urea Nitrogen

Casp3 – Caspase 3 gene

Casp12 - Caspase 12 gene

CC – Control maternal diet and Control offspring diet

Ccl2 - Monocyte chemoattractant protein 1 gene

CD68 - Cluster of Differentiation 68

cDNA – complementary DNA

CHD – Coronary Heart Disease

CKD – Chronic Kidney Disease

CO – Control maternal diet and Obese offspring diet

CO_2 – Carbon dioxide

CRH - corticotropin-releasing hormone

CT – Cycle threshold

CVD – Cardiovascular Disease

dH_20 – distilled water

DNA - Deoxyribonucleic Acid

DOHaD - Developmental Origins of Health and Disease Hypothesis

E – Embryonic day

EDTA - Ethylenediaminetetraacetic acid

ELISA - enzyme linked immunosorbent assay

EMT - epithelial to mesenchymal cell transition

ER – Endoplasmic Reticulum

ERK1/2 - phosphorylated 44/42 mitogen activated protein kinase

ESRF – End Stage Renal Failure

Eya1 - EYA Transcriptional Coactivator And Phosphatase 1 gene

F4/80 - EGF-like module-containing mucin-like hormone receptor-like 1

FFA – Free Fatty Acids

Fn1 - Fibronectin gene

g – grams

Gapdh - Glyceraldehyde 3-phosphate dehydrogenase gene

GBM – glomerular basement membrane

GDM – Gestational Diabetes Mellitus

GDNF - Glial derived neurotrophic factor

Gdnf - Glial derived neurotrophic factor gene

GFR – Glomerular Filtration Rate

GFRA1 - Glial derived neurotrophic factor receptor alpha

Gfra1 - Glial derived neurotrophic factor receptor alpha gene

GLUT-1 – Glucose transporter 1

GR – Glucocorticoid receptor

GWG – Gestational Weight Gain

H_2O – Water

HAPO - Hyperglycaemia and Adverse Pregnancy Outcome

HDL – High Density Lipoprotein

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIF1A – Hypoxia Inducible Factor 1 alpha

*Ho*x – Homeobox genes

Hox11 – T cell leukemia homeobox 1 gene

HPA - Hypothalamic–Pituitary–Adrenal

Hprt - Hypoxanthine Phosphoribosyltransferase gene

IgAN - immunoglobulin A nephropathy

IGF – Insulin-like growth factor

IPGTT – Intraperitoneal Glucose Tolerance Test

IQR – Inter Quartile Range

IRS-1 – Insulin Receptor Substrate 1

IUGR – Intra Uterine Growth Restriction

kDa - kilodalton

kg – kilograms

KIM-1 – Kidney injury molecule 1

Kim1 - Kidney injury molecule 1 gene

l - litre

LBW – Low Birth Weight

LDL – Low Density lipoprotein

LGA – Large for Gestational Age

Lim1 - LIM homeobox 1 gene

LPL – lipoprotein lipase

Lpl - lipoprotein lipase gene

MAPK – Mitogen Activated Protein Kinase

MC3/4R - melanocortin receptor 3/4

MCP-1 – Monocyte Chemoattractant Protein 1

mg – milligrams

ml – millilitres

mm^2 – millimetres squared (area)

mmol – millimoles

MMP-2 - Matrix metalloproteinase 2

Mmp2 - Matrix metalloproteinase 2 gene

MOPS - 3-(N-morpholino) propanesulfonic acid

mRNA – messenger RNA

mTOR - mammalian target of rapamycin

n – Sample number

NAG - N-acetyl-b-D-glucosaminidase

NF-κB - nuclear factor kappa-light-chain-enhancer of activated B cells

NFDM – Non-fat Dried Milk

ng – nanograms

NHS – National Health Service

nm – wavelength

OC – Obese maternal diet and Control offspring diet

Ob-Ex – Obese maternal diet with exercise during gestation

OO – Obese maternal diet and Obese offspring diet

P1 – First pregnancy

P2 – Second pregnancy

p38 MAPK - phosphorylated p38 mitogen activated protein kinase

PAS – Periodic acid-Schiff

Pax2 - paired box 2 gene

Pgc-1 α - peroxisome proliferator-activated receptor γ co-activator-1α gene

PN – Postnatal day

pmol – picomoles

PVDF - Polyvinylidene Difluoride

qPCR – Quantitative Real Time Polymerase Chain Reaction

RAS – renin-angiotensin system

Ren1 – Renin gene

RET - Receptor tyrosine-protein kinase

Ret - RET receptor tyrosine kinase gene

RNA - Ribonucleic Acid

ROS – Reactive oxygen species

S – Standard

Sdha - Succinate Dehydrogenase Complex Flavoprotein Subunit A gene

SDS - Sodium Dodecyl Sulfate

SDS-PAGE - SDS polyacrylamide gel electrophoresis

SEM – Standard Error of the Mean

SGA – Small for Gestational Age

SIX2 - sine oculis-related homeobox 2

Six2 - sine oculis-related homeobox 2 gene

Srebp1 - Sterol Regulatory Element Binding Transcription Factor 1 gene

STZ – Streptozocin

T2DM – Type 2 diabetes melitis

TBS – Tris-buffered Saline

TBS/T – Tris-buffered Saline with Tween 20

TD-NMR – Time Domain- Nuclear Magnetic Resonance

TEMED – Tetramethylethylenediamine

TGF-β – Transforming growth factor beta

UB – Ureteric Bud

UK – United Kingdom

US – United States of America

V1AR - vasopressin receptor 1A

V1BR - vasopressin receptor 1AB

V2R - vasopressin receptor 2

WHO – World Health Organisation

Wnt4 - Wnt family member 4 gene

Wnt9b - Wnt family member 9b gene

Wnt11 - Wnt family member 11 gene

Wt1 – Wilms tumour 1 gene

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Chapter 1. General Introduction

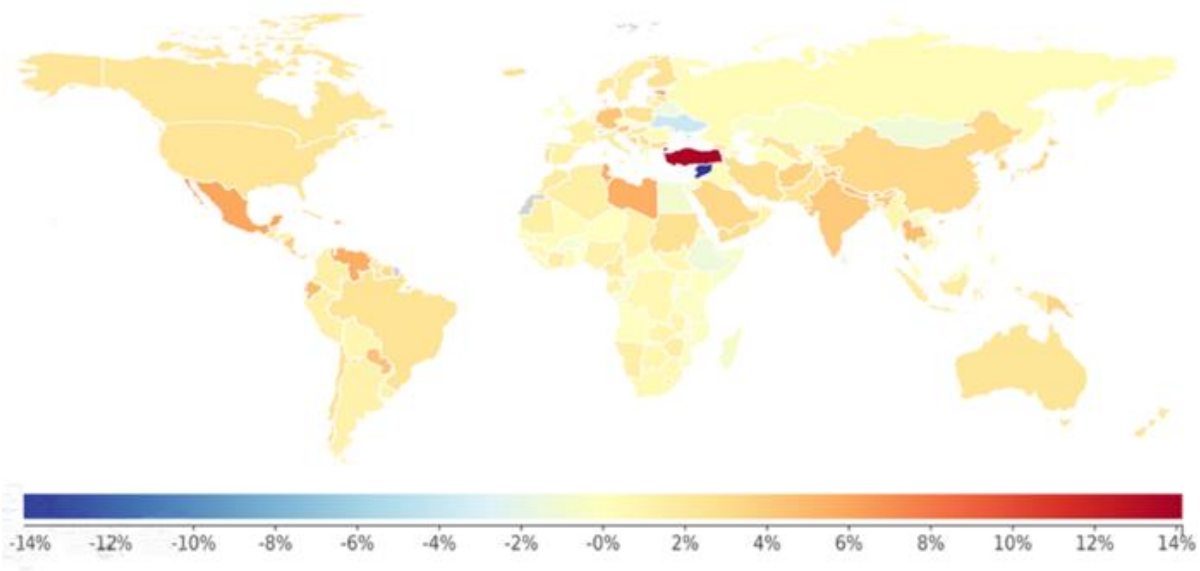
1.1 Renal disease: a new arising epidemic

Chronic Kidney Disease (CKD) is defined as a reduced glomerular filtration rate (GFR), increased albumin excretion in the urine, or both (Jha et al. 2013). The disease can be classified into 5 stages as shown in Table 1.1. It is one of the fastest growing diseases, affecting over 10% of the global population (Hill et al. 2016). Deaths from CKD increased 83% globally between 1990 and 2010 (Luyckx et al. 2013), increasing its ranking from the 27th to the 18th most common cause of mortality globally (Jha et al. 2013). During the same time period the number of years lived with disability due to CKD increased by 20% (Lopez et al. 2014). Figure 1.1 displays the global increase in mortality due to CKD over the last couple of decades.

Table 1.1 *The stages of CKD*

Stage	**GFR (mL/min/1.73m^2)**	**Clinical characteristics**
1	≥90	Normal kidney function. Urine findings or structural abnormalities or genetic trait point to kidney disease.
2	60-89	Mildly reduced kidney function. Other findings (as in stage 1) point to kidney disease.
3	30-59	Moderately reduced kidney function.
4	15-29	Severely reduced kidney function.
5	<15	Very severe or End Stage Renal Failure (ESRF)

Adapted from The Renal Association. (Anon n.d.)



Annual percentage change per country in deaths attributed to chronic kidney disease from 1990 to 2013. Source: Institute for Health Metrics and Evaluation, University of Washington.

Figure 1.1. **The annual percentage change in deaths per country attributed to chronic kidney disease from 1990 to 2013**. Taken from (Stanifer n.d.).

The global increase in CKD mirrors the obesity epidemic. Worldwide obesity has more than doubled in prevalence since 1980. In 2014, 13% of over 18's (11% of men and 15% of women) were obese, and 39% (38% of men and 40% of women) were overweight (WHO 2016). In the UK and US obesity prevalence is much higher, with 27% and 37% of adults estimated to be obese respectively (WHO 2013; Ogden et al. 2015). This is attributed to shifts in dietary patterns to energy dense foods, rich in fats and sugars, and decreases in levels of physical activity.

Obesity drives two leading causes of CKD worldwide: hypertensive glomerulosclerosis and type 2 diabetic nephropathy, which together now account for almost three-quarters of end-stage renal disease (De Vries et al. 2014) (See Figure 1.2). Obesity is the central feature of the metabolic syndrome, defined as a sequelae of inter-linked cardio-metabolic disorders including dyslipidaemia, hypertension and type 2 diabetes (T2DM). Each of these traits poses an independent risk to renal function such that the risk of CKD rises with the number of traits an individual possesses (De Vries et al. 2014). Importantly, there is evidence that body mass index (BMI) positively correlates with the incidence of End Stage Renal Failure (ESRF) even when controlling for other key risk factors including diabetes, blood pressure and dyslipidaemia (De Vries et al. 2014).

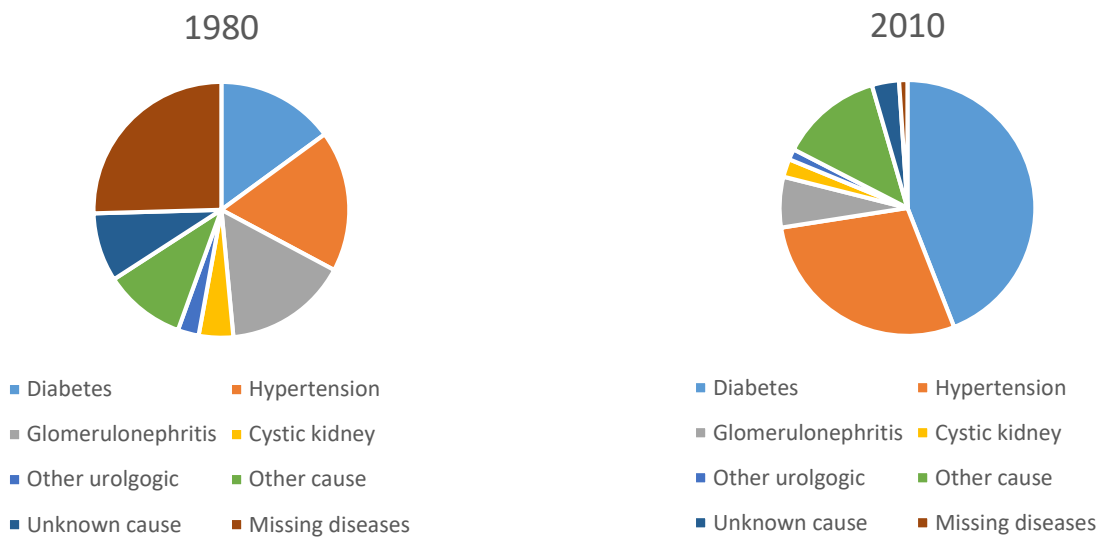


Figure 1.2. **The causes of new cases of ESRF in the US in 1980 and in 2010.** Adapted from data taken from (Health, United States, 2011: table 51. End-stage renal disease patients, by selected characteristics: United States, selected years 1980–2010.). (National Institute of Diabetes and Digestive and Kidney Disease 2014).

Individuals with CKD have a 2-5 fold increase in the risk of death, hospitalisation or major vascular events compared with people who have normal renal function (Lopez et al. 2014). Cardiovascular diseases are the leading cause of mortality in CKD patients, responsible for 50% of deaths. Remarkably, there is some evidence that the combination of microalbuminuria (where small amounts of albumin leak into the urine) and mild renal insufficiency confers a greater risk for cardiovascular events than that observed in patients with a coronary heart disease with normal renal function (Trevisan 2006). Less than half of CKD patients actually go on to develop ESRF since most die from atherosclerosis beforehand (Orli et al. 2014). The above epidemiological observations demonstrate the severity of CKD since prognostic outcomes are poor. Furthermore, these observations highlight the complexity of the aetiology of CKD; risk factors such as a failing heart can be both a cause and a consequence of the disease.

Perhaps due to the complex nature of both the causes and progression of the disease, CKD costs the NHS in England more than £1.4bn per year, more than breast, colon and skin cancer combined (Kerr et al. 2012). With the incidence of metabolic syndrome projected to continue to increase globally (Couser et al. 2011), CKD will likely grow as a health and economic burden.

1.2 Causes of Chronic Kidney Disease

1.2.1 Obesity and the metabolic syndrome

Obesity is the main driving force behind T2DM and hypertension. Obesity accounts for around 78% and 68% of essential hypertension in men and women respectively according to data from the Framingham cohort study (El-atat et al. 2004). Obesity is thought to cause hypertension by increasing renal tubular sodium reabsorption and causing a hypertensive shift of renal-pressure natriuresis in the kidney. This occurs via activation of pathways including the sympathetic nervous system and the renin-angiotensin system (RAS) (Maric-Bilkan 2013). Hypertension and diabetes are the most common causes of CKD. However, obesity is also associated with other factors such as dyslipidaemia. All of these factors can promote CKD independently and synergistically. Indeed, studies have shown that kidney outcomes are improved when hyperglycaemia, hypertension and dyslipidaemia are targeted separately (Alexander et al. 2009). Ultimately, all these factors promote fibrosis of the kidney leading to the loss of functional nephrons and a steady decrease in renal function culminating in ESRF. The mechanisms by which these factors contribute to CKD are discussed below in turn.

Hormonal changes as well as low grade inflammation are also thought to play a key role in the aetiology of obesity related renal disease (de Jong et al. 2002). Obesity is a low grade inflammatory disease [adipocytes are known to produce cytokines (de Jong et al. 2002)] which can have a notable influence on renal function by promoting the infiltration of macrophages into renal tissue (Maric-Bilkan 2013). Macrophages are a primary source of pro-inflammatory mediators in the kidney which promote the fibrogenesis and albuminuria associated with obesity related glomerulopathy (Maric-Bilkan 2013). Insulin resistance (discussed below) and increased leptin levels are also characteristic of obesity and are associated with renal disease. Leptin appears to stimulate transforming growth factor beta (TGF-β) production by glomerular endothelial and mesangial cells, initiating excessive extracellular matrix deposition and leading to glomerulosclerosis (de Jong et al. 2002; Maric-Bilkan 2013).

Obesity related glomerulopathy is characterised by focal segmental glomerulosclerosis. Additionally, individuals with obesity related glomerulopathy show glomerulomegaly (glomerular hypertrophy) with mesangial cell proliferation, matrix accumulation, and a decreased density of podocytes which tend to be detached from the glomerular basement membrane (GBM) and hypertrophied (De Vries et al. 2014).

1.2.1.1 Diabetes

Diabetic nephropathy is the most common cause of CKD since 30-40% of diabetic patients go on to develop the complication. Hyperglycaemia is the main factor driving diabetic nephropathy since the disease is unable to develop without it (Schena 2005). However, other factors associated with diabetes such as dyslipidaemia (discussed below) are also important in the progression of the disease, highlighting that, like most renal diseases, diabetic nephropathy has a complex aetiology.

1.2.1.1.1 Hyperglycaemia

Hyperglycaemia induces damage to the kidneys both by haemodynamic alterations and by direct damage. Hyperfiltration, where glomeruli filter excessive amounts of blood and produce excessive pro-urine, is a characteristic of pre-diabetes. It has been demonstrated that fasting glucose is associated with hyperfiltration independent of age, sex, body mass index, blood pressure, smoking status and insulin levels (Palatini 2012). There is now direct evidence that glucose stimulates the release of renin from the juxtaglomerular apparatus (Toma et al. 2008), initiating the RAS cascade which promotes afferent arteriole dilatation and efferent arteriole constriction leading to hyperfiltration (Helal et al. 2012). Hyperfiltration induces glomerular hypertrophy and hypertension leading to functional changes such as shear stress and increased permeability of the glomerular basement membrane. This allows albumin to leak through the glomerular basement membrane and encourages the production of extracellular matrix within the glomerulus leading to mesangial cell expansion, glomerular basement membrane thickening and glomerulosclerosis (Schena 2005). The presence of albumin and associated cytokines within the tubules also stimulates inflammation leading to tubulointerstitial fibrosis (Zeisberg & Neilson 2010).

Hyperglycaemia induces direct damage by promoting both resident and non-resident cells to produce cytokines, humoral mediators and growth factors. These promote structural changes such as increases in deposition of extracellular matrix proteins (collagens, laminin and fibronectin), and also increase the permeability of the GBM. A key factor in the pathogenesis of diabetic nephropathy is TGF-β. TGF-β is upregulated by many factors associated with diabetes, including Angiotensin II and advanced glycosylation end products, and it initiates the expression and translocation of glucose transporter 1 (GLUT-1) to cell membranes, allowing glucose to freely enter cells and thus perpetuating a vicious pathological cycle. TGF-β also stimulates extracellular matrix deposition which leads to glomerulosclerosis and tubulointerstitial fibrosis (Schena 2005).

1.2.1.1.2 Hyperinsulinemia/insulin resistance

Hyperinsulinaemia/insulin resistance is known to be a key factor leading to renal damage. Epidemiological studies have demonstrated that insulin resistance is an independent predictor of CKD and that higher fasting insulin is associated with age related decline in renal function (De Vries et al. 2014). Hyperinsulinaemia promotes increased intra-glomerular pressure, hyperfiltration and hypertrophy through endothelial dependent vasodilatation of the glomerular blood vessels (Bagby 2004; Hale & Coward 2013). These haemodynamic changes promote damage due to shear stress (discussed in section 1.2.1.2). Additionally, insulin can stimulate glomerular hypertrophy directly through the action of insulin growth factor 1 (IGF-1) (Bagby 2004). Furthermore it has been shown *in vivo* that insulin can directly lead to the production of extra-cellular matrix proteins by mesangial cells (Abrass et al. 1994). It's also important to note that whilst hyperinsulinaemia may serve to maintain the actions of insulin in insulin resistant organs, in insulin sensitive organs, abundant insulin would promote excessive uptake of glucose and lipids which could promote damage. For example, mesangial lipid accumulation via IGF-1 has been associated with loss of contractile function and might contribute to loss of function in the glomerulus (D'Agati et al. 2016). Additionally, podocytes respond to insulin through mammalian target of rapamycin (mTOR) and remodel their actin cytoskeleton to prepare the GBM for an increase in GFR postprandially (Hale & Coward 2013). The ectopic accumulation of lipids within podocytes through the excessive activation of mTOR can promote podocyte specific insulin resistance, undermining the integrity of the GBM and initiating glomerulosclerosis and proteinuria (Hale & Coward 2013; De Vries et al. 2014).

Hyperinsulinaemia and insulin resistance also have important consequences in the tubules. The kidney is the second most important organ for gluconeogenesis after the liver. Insulin suppresses gluconeogenesis in the kidney. In obese patients, renal gluconeogenesis rises to levels comparable to that seen in the liver after fasting, and in T2DM, the kidney is thought to contribute glucose to the circulation causing hyperglycaemia. This suggests that insulin resistance in the kidney tubular cells enhances renal gluconeogenesis. Increased gluconeogenesis eventually leads to increased tubular atrophy and interstitial fibrosis (De Vries et al. 2014).

1.2.1.2 Hypertension

Hypertension accounts for a large proportion of CKD cases. The importance of hypertension in contributing to renal disease is highlighted by the fact that renal injury usually only occurs in diabetes and obesity when hypertension is present, and the importance of controlling blood pressure to prevent renal decline in diabetic patients is well known (Ravera et al. 2006; Maric-Bilkan 2013).

Systemic hypertension contributes to renal injury primarily through glomerular hyperfiltration. This occurs through vasodilatation of the afferent arterioles leading to increased pressure within the capillaries and causing individual increases in GFR (D'Agati et al. 2016). Increased hydrostatic pressure in the glomerular capillaries stresses the capillary walls causing expansion of the basement membrane and leading to glomerular hypertrophy. Increased hyperfiltration also places shear stress on podocytes leading to podocyte loss and detachment from the basement membrane (D'Agati et al. 2016). Because podocytes are incapable of regenerative cell replication, the remaining podocytes are then put under even more functional strain and undergo hypertrophy to maintain the integrity of the GBM (Kriz & LeHir 2005). Eventually, when the podocytes can no longer cope with this strain, they detach from the GBM. This allows uncovered areas of the GBM to attach to parietal cells, and proteins to leak through the barrier, initiating inflammatory and fibrogenic events leading to glomerulosclerosis (Kriz & LeHir 2005; D'Agati et al. 2016). Once the glomerulus is damaged sufficiently to undermine the integrity of the GBM, the proteins and associated cytokines that leak through into the tubules are thought to be one of the main things initiating inflammation and fibrogenesis within the tubules (Zeisberg & Neilson 2010; Hodgkins & Schnaper 2012). Additionally, hydrodynamic forces along the tubules themselves are pro-fibrotic. An increase in GFR due to hypertension would place shear stress on the tubules initiating tubulointerstitial fibrosis (Zeisberg & Neilson 2010).

Whilst systemic hypertension contributes to renal disease by promoting hyperfiltration, it's important to note that this process is bidirectional and the kidney also has an important role in maintaining hyperfiltration and promoting hypertension. Obesity and diabetes are associated with an activation of renal mechanisms that promote hypertension. The RAS is directly activated in early diabetes and obesity as mentioned above. The RAS promotes hyperfiltration by vasodilatation of the afferent arterioles, but also promotes increased tubular sodium reabsorption leading to hypertension (Palatini 2012). The so-called "tubular hypothesis" may also promote hypertension and hyperfiltration associated with obesity and diabetes. This involves increased uptake of sodium by the proximal tubules via the sodium-glucose cotransporter-2, which presumably is upregulated to reclaim excess glucose in the tubules. This decreases salt delivery to the macula densa (epithelial cells in the junction of the thick ascending limb and distal convoluted tubule and adjacent to the glomerulus), which perceives the decrease in salt as an overall decrease in renal perfusion and GFR. A maladaptive tubuloglomerular feedback mechanism then stimulates factors which promote increases in GFR (Sasson & Cherney 2012). These mechanisms lead to positive feedback between hypertension and declining renal function leading to CKD. Additionally, the urotensin system within the kidney has recently been shown to be important for the development of spontaneous hypertension. In humans, hypertension has been shown to be correlated with urotensin II concentrations in plasma and urine.

Consistently, in the spontaneous hypertensive rat (where renal abnormalities have been shown to be responsible for the development of hypertension) it was demonstrated that urotensin related peptide decreased GFR in the pre-hypertensive phase, whilst an urotensin II receptor antagonist increased GFR, urine flow and sodium excretion. Urotensin related peptide expression was also increased in the spontaneously hypertensive rat (Forty & Ashton 2013). These observations suggest that the urotensin system, by increasing sodium retention in the kidney, may be involved in the pathogenesis of hypertension.

1.2.1.3 Dyslipidaemia

There is evidence that the presence of focal segmental glomerulosclerosis and glomerular hypertrophy in obesity is not correlated with the intensity of obesity *per se*, but with serum triglycerides and the renal deposition of lipids (De Vries et al. 2014). The infiltration of fat into the kidney (kidney steatosis) is also in close association with fat deposition in the liver (liver steatosis) and with unhealthy indices of metabolic disease (De Vries et al. 2014). This suggests that dyslipidaemia plays an important role in CKD resulting from obesity. Patients with unhealthy obesity show fat deposition in mesangial cells, podocytes and tubular cells. As mentioned above, lipid deposition in mesangial cells can cause them to lose their contractile ability, contributing to glomerular hyperfiltration and hypertrophy, whilst lipid deposition in podocytes leads them to become insulin resistant, undermining the integrity of the GBM and contributing to albuminuria and glomerulosclerosis (D'Agati et al. 2016). In addition, lipids can become trapped in the extracellular matrix of cells where they undergo oxidation leading to reactive oxygen species (ROS) production. Macrophages phagocytise oxidised lipids and transform into foam cells. These in turn produce more cytokines to recruit more macrophages. The resulting inflammation also promotes sclerosis (Trevisan 2006).

Tubular uptake of free fatty acids (FFAs) increases in obesity and occurs in parallel with both albuminuria (luminal side) and plasma FFA concentrations (basolateral side). Although some of these fatty acids are utilised for tubular transport energy demands, a substantial amount are incorporated into lipid droplets. Studies show that excess FFA deposition is another factor which promotes tubulointerstitial injury (De Vries et al. 2014). Furthermore, the absorption of FFA bound albumin into proximal tubular promotes insulin resistance, and so leads to enhanced gluconeogenesis as described above, again promoting tubulointerstitial fibrosis (De Vries et al. 2014).

1.2.2 Glomerulonephritis

Glomerulonephritis can be defined as inflammation of the glomeruli caused by an immune response. Although diabetes and hypertension now account for many of the cases of CKD in the developed world, glomerulonephritis (not caused by either of these factors) still accounts for a large proportion of kidney disease (Barsoum 2006; Couser et al. 2011). This is especially true in the developing world, reflecting the high prevalence of bacterial, viral and parasitic infections in these areas (Barsoum 2006). The causes of glomerulonephritis are diverse, complicated and often not understood. The disease can occur "primarily" due to intrinsic factors within the kidney. For example, immunoglobulin A nephropathy (IgAN) is the most common cause of primary glomerulonephritis, affecting up to 1.3% of the population (Gharavi et al. 2000). It involves deposition of IgA-containing immune complexes within the kidney that leads to proliferation of the glomerular mesangium and blood and albumin in the urine. The pathogenesis of IgAN is unknown, although recent evidence demonstrates that genetics play a key role (Gharavi et al. 2000; Suzuki et al. 2011). Glomerulonephritis may also occur "secondarily" due to e.g. viral/bacterial/parasitic infections, chronic autoimmune diseases such as lupus erythematosus, drugs and malignancy (Mason & Pusey 1994). The disease can also be broadly classified into proliferative (involving proliferation of cells within the glomerulus), and non-proliferative (characterised by non-proliferation of cells in the glomerulus). All types of glomerulonephritis can promote nephritic syndrome (blood and albumin in the urine, a decrease in urine production and hypertension) and nephrotic syndrome (albumin in the urine, low blood albumin levels, high blood lipid levels and edema) (Mason & Pusey 1994). The types of glomerulonephritis and how they are classified are reviewed in (Mason & Pusey 1994).

1.2.3 Polycystic kidney disease

Polycystic kidney disease is the most common form of heredity kidney disease (Reule et al. 2014). In the US, it is responsible for roughly 5% of ESRF cases (Reule et al. 2014). The disease is characterised by fluid filled cysts in the kidney and other organs. Within the kidney, the cysts originate from epithelia of the nephrons and renal collecting system (Igarashi 2002). Polycystic kidney disease can either be an autosomal dominant trait (fairly common and found in children and adults) or recessive trait (rare and found in neonates and children) (Igarashi 2002). Both forms of the disease involve the gradual growth of intra-renal cysts which leads to kidney enlargement and a progressive decline in renal function (Halvorson et al. 2010).

1.2.4 Causes of CKD: genetics vs. the environment

It is clear from the causes of CKD discussed above, that the environment is a large determinant of renal disease. Indeed, the largest causes of CKD are hypertension and T2DM that are considered mainly lifestyle driven diseases. It's also clear that kidney disease can be entirely genetically determined, as in the case of polycystic kidney disease. However, it's also important to consider that the pathogenesis of renal disease is often complicated, and genetic and environmental factors may interact to determine the course and severity of disease. For example, a higher incidence of hypertension and glomerulosclerosis was found in children with IgAN (a disease where genetics are known to play a role) who were growth restricted at birth (Zidar et al. 1998). This observation demonstrates that the quality of the early life environment can affect the outcome and severity of a partly genetically determined disease. Additionally, the early life environment has been shown to interact with the later life environment in determining the severity of renal disease. For example, in patients with unilateral renal agenesis (where one kidney fails to develop), obesity is known to accelerate renal damage and dysfunction (Kett & Denton 2011). Importantly, a substantial number of CKD cases have no known cause and the proportion of cases of CKD that can't be explained by hypertension or diabetes is significantly higher in developing countries (Couser et al. 2011). It is known that disadvantaged populations have experienced a drastic increase in CKD prevalence in recent years and it has been suggested that this increase could be due to the fact that these populations experience poor intrauterine environments more frequently (Nelson 2003). Therefore, the quality of the intrauterine environment is a likely, albeit less recognised cause of later CKD (Ritz et al. 2011).

1.3 The Developmental Origins of Health and Disease

There is now substantial evidence that the early life environment plays a significant role in the quality of organ development as well as function and subsequently, the risk of disease in later life. Therefore, to prevent diseases such as CKD, optimisation of the early life environment must be considered as an important period to target interventions.

1.3.1 Fetal origins

The first suggestion that the fetal environment could influence later life health was posed by Barker and colleagues in 1989. Tracing the lives of men born in Hertfordshire between 1911 and 1930, they found that men with low birth weights, a proxy for the quality of the *in utero* environment, had the highest mortality rates from ischemic heart disease (Barker et al. 1989). Similarly, it was discovered in a cohort of men and women from Lancashire, that a poor *in utero* environment was associated with increased systolic and diastolic blood pressure. Birth weight negatively correlated and placental weight positively correlated with hypertension. Importantly, these associations were independent of lifestyle influences (Barker et al. 1990). Additionally, Eriksson and colleagues supported the hypothesis further by uncovering the same relationship in men from the Helsinki birth cohort. Death from coronary heart disease was more likely in men who were thin at birth or had a low birth weight (LBW), and this risk was further increased by accelerated growth during childhood (Eriksson et al. 1999).

1.3.2 Thrifty phenotype

The first notion that the effects of the *in utero* environment could extend beyond cardiovascular disease came once again from the Hertfordshire cohort. There was an inverse correlation between birth weight and glucose intolerance such that men with the lowest birth weights had the highest glucose levels (Hales et al. 1991). Importantly, this association was independent of current body weight, suggesting early life "programming" of glucose homeostasis. These observations led Hales and Barker to propose the "thrifty phenotype" hypothesis. They proposed that in a nutrient deficient *in utero* environment, exposed offspring would be forced to be "thrifty" with the resources available to them for development. In this scenario, resources would be diverted to integral organs such as the brain and away from organs such as the pancreas and kidneys. In addition it was proposed that the fetus developed a "thrifty metabolism" that was efficient at storing nutrients when they were available. These adaptations may be beneficial in the short term, ensuring survival until birth, but may trade off with later life health when organs like the pancreas are challenged. In this manner, a poor *in utero* environment could promote increased risk of disease in adulthood.

1.3.3 Predictive adaptive response hypothesis

Both the fetal origins and thrifty phenotype hypotheses assume that later life detriments arising in individuals exposed to a poor fetal environment are "accidental" consequences of adaptations made by the fetus to ensure short term survival. However, Mark Hanson and Peter Gluckman realised that

not all adaptations made by the fetus in response to early life cues confer advantages in the short term, but instead are advantageous much later on in adult life. This led them to develop the predictive adaptive response hypothesis which states that some adaptations made in early life may be predictive of the future environment, increasing fitness in adulthood (Gluckman et al. 2005). Hanson and Gluckman realised the implications of these predictive adaptive responses for adaption and evolution, but they also hypothesised they would be important for human disease. They argued that if the *in utero* environment is matched with that of the later life environment, predictive responses should be beneficial and increase fitness. However, if the *in utero* environment is mis-matched with that of the later life environment, early responses would confer no fitness advantages and may actually be maladaptive, for instance by promoting disease. Specifically, in humans Hanson and Gluckman argued that predictive adaptive responses may be partially responsible for the increased incidence of cardiovascular and metabolic disease seen in recent years. This is because growth in the fetal period is constrained by factors such as female pelvic diameter, and therefore the fetus is also constrained in its predictive capabilities. Particularly in the developed world, rapid environmental changes have led to increased levels of postnatal resource abundance beyond the scope of what the fetus can predict given these constraints, rendering predictive adaptive responses inadequate in the modern enriched world.

1.3.4 DOHaD

The Developmental Origins of Health and Disease Hypothesis was developed to encompass and extend the Barker, thrifty phenotype and predictive adaptive response hypotheses. Evidence in the field highlighted that both conception and postnatal life are also important for later life outcomes. Therefore the term "developmental" was adopted to emphasise that the whole period of plasticity, extended beyond fetal life, and is important in determining the adult phenotype. Earlier hypotheses also emphasised disease as an outcome of an adverse early life environment, so the term "health" was also introduced to highlight the idea that general health is influenced by the early life environment (Gluckman et al. 2006).

1.3.4.1 DOHaD – Under-nutrition

There is now ample epidemiological evidence supporting the DOHaD hypothesis, much of which comes from studies of maternal under-nutrition. The Dutch hunger winter was a period of famine from 1944-1945 in the Netherlands where resources were cut off from the western part of the Netherlands due to German occupation. Due to thorough medical and rationing records from the time,

the famine has presented a unique opportunity for researchers to directly address the impact of maternal under-nutrition during gestation on offspring health. It has been demonstrated that individuals exposed to famine during early gestation had an increased atherogenic profile, increased risk of obesity and metabolic disease as well as a 3-fold increase in incidence of CVD (Ravelli & Osmond 1999; Roseboom et al. 2000; Roseboom et al. 2000). Those exposed to famine during late gestation displayed impaired glucose tolerance and an increased risk for T2DM (Ravelli et al. 1998; Roseboom et al. 2001). Importantly, these associations were independent of birth weight, emphasising a direct impact of early life nutritional deprivation on later life cardio-metabolic health. These studies also underline the importance of the timing of a developmental insult on the nature of later life health and disease.

Monozygotic and dizygotic twin studies have provided the opportunity to separate the impacts of the maternal environment from genetic influences. Again, these studies support the DOHaD hypothesis, highlighting that variation in characteristics such as serum cholesterol, glucose, insulin, leptin and blood pressure are mainly derived from the nature of fetal growth (Vaag & Poulsen 2007; Jermendy et al. 2011; Touwslager et al. 2013). For example, in both monozygotic and dizygotic twin pairs, it has been observed that the twin with the lower birth weight is more likely to develop impaired glucose tolerance and T2DM (Poulsen et al. 1997). These studies emphasise the importance of the early life environment in determining the quality of later life health.

1.3.4.2 DOHaD – Over-nutrition

More recently, with obesity prevalence rising rapidly across the globe, the field has shifted towards investigating the impact of early life over-nutrition on later life health. Several studies have now highlighted a positive correlation between maternal BMI or gestational weight gain (GWG) and adverse cardio-metabolic measures in exposed individuals including increased adiposity and blood pressure, in both in childhood (Lawlor et al. 2004) and early adulthood (Mamun et al. 2009), increased glucose, insulin and insulin resistance (Gaillard et al. 2015) and increased rates of T2DM (Dabelea et al. 2008). Importantly, there is evidence that these adversities lead to poor outcomes in later adulthood. In 34-61 year olds exposed to maternal obesity, hospital admissions due to cardiovascular events were increased along with all-cause mortality (Reynolds et al. 2013). It could be argued that some cardio-metabolic abnormalities arise in offspring exposed to maternal obesity due to the inheritance of genes associated with the obese phenotype. However, it has been observed that children born to mothers following weight loss due to bariatric surgery show a 50% decrease in obesity compared to their siblings born prior to surgery (Kral et al. 2006). This interventional study solidifies the role of the early life obesogenic environment *per se* on the health of future generations.

Furthermore, siblings born to obese mothers before and after bariatric surgery show differences in the methylation of genes related to insulin, diabetes and leptin signalling as well as genes involved in inflammation and vascular disease (Guénard et al. 2013; Berglind et al. 2016). These findings emphasise epigenetic alterations as a plausible mechanism linking exposure to an early life obesogenic environment to an impaired later life cardio-metabolic phenotype.

Maternal gestational diabetes (GDM) as a risk factor for poor offspring health has also gained attention in recent years. GDM shows similarities with maternal obesity in terms of offspring outcomes. GDM increases the risk for offspring central adiposity (Lawlor et al. 2010), increased BMI, blood pressure and an adverse glucose profile (Bunt et al. 2005; Malcolm et al. 2006; Tsadok et al. 2011). Importantly, exposure to diabetes during early life development increases the risk for development of metabolic syndrome (Boney et al. 2005) and T2DM (Franks et al. 2006; Clausen et al. 2008; Dabelea et al. 2008) such that a vicious cycle may perpetuate between succeeding generations. Notably, increased rates of exposure to GDM is suggested as the causal factor involved in the drastic increase in T2DM rates seen among disadvantaged populations (Dabelea et al. 1998; Osgood et al. 2011). Dabelea and colleagues assessed the prevalence of diabetes in siblings born to mothers before and after the onset of diabetes and showed that individuals exposed to maternal diabetes were significantly more likely to develop the disease themselves. In further analysis, the presence of paternal diabetes had no association with offspring diabetes risk (Dabelea et al. 2000). This study highlights that, like a maternal obesogenic environment, a diabetic milieu during pregnancy can profoundly impact upon later life offspring health independently of genetic factors.

Conditions of early life over-nutrition, such as maternal diabetes or obesity, lead to similar phenotypes within exposed individuals as early life under-nutrition. Therefore, the risk of disease is U shaped, with both extremes of early life nutrition increasing the probability of poor later life health. One phenotype that has been seen as a result of both early life under and over-nutrition is renal dysfunction.

1.4 The developmental origins of renal health

Although T2DM underlines most cases of CKD globally, a substantial amount of variation in terms of renal outcomes in response to T2DM exists between ethnic groups. When controlling for the prevalence of diabetes within populations, black Americans are 4 times more likely and Mexican Americans are 2.5 times more likely to get ESRF than non-Hispanic Whites (Nelson 2003). Likewise,

Asians in the UK are 14 times more likely to develop diabetic ESRF than Whites in the same area, whilst the rate of the disease in diabetic Pima Indians is 14 times higher than the estimated rate in the U.S diabetic population aged 45-64 years (Nelson 2003). Poor environments more likely unify these populations than for example, genetic factors. This raises the possibility that this disparity in renal disease prevalence is may be partially explained by the nature of early life development, since disadvantaged populations experience a higher frequency of poorer *in utero* environments. Indeed, conditions leading to adverse early life environments including both maternal under-nutrition and over-nutrition have been shown to impair renal growth and later life renal health in exposed individuals.

1.4.1 Maternal under-nutrition/IUGR

Low birth weight infants are defined as those who weigh ≤2500 g at birth. These individuals can be further divided into preterm (those born before 37 weeks after the last menstrual period), appropriate for gestational age (AGA), or small for gestational age (SGA). In the developed world, preterm births mostly contribute to LBW outcomes whereas IUGR causes the majority of LBW cases in developing countries (Hershkovitz et al. 2007). In the US, the number of LBW infants has risen in recent years owing to an increase in the number of complicated pregnancies reaching term as well as improved postnatal survival (Hershkovitz et al. 2007). The likelihood of LBW outcomes is determined by the quantity and quality of nutrients reaching the developing fetus, such that poor maternal nutrition will increase the risk along with factors that disturb nutrient transfer across the placenta such as smoking and hypertension. The space available for fetal growth also affects birth weight, such that women of short stature or who were SGA themselves are more likely to have babies of LBW (Luyckx et al. 2013). All of these factors are in turn associated with poor maternal socioeconomic status.

Several studies have shown a relationship between LBW and altered renal morphology. Using ultrasound, Konje and colleagues found that babies who were SGA during the third trimester had thinner kidneys than AGA babies (Konje et al. 1996), suggesting a decrease in overall renal growth. A decrease in renal growth pertaining to a poor *in utero* environment is associated with reduced nephron number. In stillborn babies and infants who died within 1 year of birth, nephron number was reduced in IUGR offspring (Hinchcliffe et al. 1992). Accordingly, glomeruli number was significantly and positively correlated with birth weight in neonates, and the glomerular volume was inversely correlated with both the number of glomeruli and birth weight (Manalich et al. 2000). The association between birth weight and nephron number is also well characterised in adults. In a study comparing autopsied kidneys from both African American and Caucasian populations, the number of nephrons

within kidneys varied 8.0 fold and was linearly correlated with birth weight. The average glomerular volume was strongly and inversely related to the number of glomeruli (Hughson et al. 2003). It has been demonstrated that the number of nephrons per kidney varies widely between individuals (ranging from 800,000–1,800,000) (Ritz et al. 2011). From the evidence collated so far, the quality of the early life environment likely contributes to this variation. These studies demonstrate a strong association between fetal under-nutrition and adverse renal development.

The inverse association between nephron compliment and glomeruli volume is thought to promote hypertension. Noting that IUGR individuals both have low nephron compliment and are prone to hypertension (Hinchcliffe et al. 1992), Brenner and colleagues hypothesised that a low nephron endowment could lead to a shift in pressure natriuresis. Specifically, they reasoned that an individual with few nephrons would have to raise systemic blood pressure in order to maintain overall filtration rates through a reduced filtration surface area. This would induce individual nephron hyperfiltration and hypertrophy, causing progressive damage to the basement membrane within the glomerulus and promoting albuminuria and nephron loss. Nephron loss would then further reinforce the rise in blood pressure (Mackenzie & Brenner 1995).

In support of this hypothesis, low nephron number and high glomerular volume have been shown to be associated with hypertension and impaired renal function. By examining the kidneys of accident victims, Keller and colleagues showed that those with hypertension had fewer nephrons of a larger volume (Keller et al. 2003). Additionally, in a study comparing Australian aborigines (a population with higher rates of renal disease, hypertension and CKD than the general population) with non-aboriginal people in one area, aborigines were found to have 30% fewer glomeruli with a 27% increase in glomerular volume. Nephron number was inversely correlated with adult height and was also related to hypertension (Hoy et al. 2006). Consistently, LBW or SGA children were found to have smaller kidneys (decreased volume and length), a higher baseline blood pressure and decreased glomerular filtration rates as compared with children of a normal birth weight. Nearly half of all LBW children were salt sensitive (defined as an increase in 24 hour blood pressure of more than 3 mm Hg on a high-salt diet as compared with a control low-salt diet) (Simonetti et al. 2008). Salt sensitivity was correlated with kidney length emphasising the association between the quality of renal growth with later life blood pressure control. These studies demonstrate a link between birth weight/*in utero* growth, nephron number and hypertension. However, since nephron endowment correlates with birth weight which itself correlates with CVD, Brenner's hypothesis is contended as it is not commonly possible to establish whether nephron deficit promotes hypertension or vice versa in humans. The association between nephron deficit and poor outcomes has been somewhat solidified by research demonstrating an association between nephron deficit and sudden infant death syndrome independent of birth

weight (Hinchcliffe et al. 1992). These studies support the notion that nephron development is strongly influenced by the quality of the *in utero* environment and can have an impact on blood pressure control.

Renal function has also been shown to be associated with the early life environment. Urinary albumin creatinine ratio was inversely related to birth weight in the Aboriginal population (Hoy et al. 1998), and albuminuria has been shown to be higher in adults who had a low ponderal index at birth (Yudkin et al. 2001). Importantly, individuals exposed to the Dutch famine during mid-gestation also showed a susceptibility to albuminuria which was independent of birth weight (Painter 2004), emphasising that under-nutrition *per se* is an important determinant of renal development and later life health. These studies highlight a link between the quality of fetal development and renal function in later life.

Finally, the likelihood of renal disease is increased in LBW individuals. Retrospective data comparing siblings in Norway showed that the risk of ESRF was increased in LBW and SGA individuals and that this association could not be explained by interfamilial factors (Ruggajo et al. 2015). Another study found a significant association between CKD and LBW in men which was independent of other lifestyle factors (Li et al. 2008). These studies emphasise that the quality of the *in utero* environment is important for later life renal health independent of genetic and lifestyle factors.

Together the evidence discussed above highlights an association between early life under-nutrition and altered renal morphology, hypertension, impaired renal function and renal disease in humans.

1.4.2 Maternal diabetes/gestational diabetes

Diabetes is a common complication in pregnancy and can be divided into pre-existing diabetes (diabetes present before pregnancy) and GDM, defined as glucose intolerance with onset or first recognition during pregnancy (Lawrence et al. 2008). GDM has been increasing rapidly in recent years and is now estimated to affect 1 in 7 pregnancies globally (International Diabetes Federation 2017). Notably, this increase has been most stark in ethnic groups, with Native American, Asian, Hispanic and African American women observed to have higher rates of GDM than white women of European descent (Ferrara 2007). These observations highlight that GDM is a particular problem in under-privileged populations. However, increasing maternal age, maternal obesity as well as a family history of diabetes are also risk factors for the development of GDM (Ferrara 2007) and, since these factors have been increasing rapidly in recent years, have likely contributed to the upsurge in GDM globally.

Epidemiological studies have demonstrated an association between exposure to maternal diabetes and poor renal health. These observations will be discussed in chapter 4, section 4.1.1.2. It's also

important to note that offspring exposed to maternal diabetes are more likely to develop T2DM themselves (Dabelea et al. 2008), creating a vicious cycle from one generation to the next. This is likely relevant for the recent upsurge in CKD prevalence. For example, the proportion of Pima Indian children exposed to diabetes has risen 4-fold in the last 30 years, leading to a 2-fold increase in diabetic cases arising from this exposure. Despite improvements in plasma glucose and blood pressure control in these individuals, the rate of proteinuria has also doubled. This is in contrast to type 1 diabetic patients in the general population, where the risk of proteinuria has decreased 30-50% since the 1930's due to improvements in blood pressure and glycaemic control. These observations suggest that the rise in proteinuria seen within the Pima Indian population may be partially due to exposure to diabetes *in utero* (Nelson 2003). This therefore highlights maternal over-nutrition as a possible contributor to the recent surge in CKD cases seen globally.

1.4.3 Maternal Obesity

Maternal obesity is increasing rapidly across the globe. In the US and the UK, over 50% of women are overweight or obese during pregnancy (Richter et al. 2015), and 20-40% of women gain more weight than is recommended during pregnancy (Thangaratinam et al. 2012). Traditionally considered a problem in developed countries, maternal obesity is rapidly becoming an issue in developing nations too. Maternal obesity increases the risk for adverse pregnancy complications and outcomes including hypertension, pre-eclampsia and caesarean delivery (Ching et al. 2016). Importantly, maternal obesity also promotes GDM (Boney et al. 2005; Ferrara 2007) and increases the likelihood for SGA (Rajasingam et al. 2009) and preterm infants (Luyckx and Brenner 2015) and, as such, likely contributes to the negative renal consequences associated with these conditions as discussed above.

Only one epidemiological study to date has investigated the impact of maternal obesity independently on offspring kidney outcomes and this will be discussed in chapter 4. As such, there is little to no information on the potential contribution of maternal obesity to the current CKD epidemic. Despite the dramatic increases in both CKD and obesity prevalence globally, and evidence that renal health can be affected by the early life environment, the kidney remains relatively unexplored territory within the DOHaD field relative to other components of the metabolic syndrome.

1.5 The importance of animal models in the study of renal programming

Epidemiological studies have come a long way in the developmental programming field and have clearly demonstrated a correlation between the quality of the maternal environment and the later life health of exposed individuals. However, they fail to address the underlying mechanisms responsible and it is difficult to demonstrate causality. Animal models are crucial to our understanding of these mechanisms since they allow for the manipulation and tight control of environmental and genetic factors. Rodent models also have the additional benefit of short generation times such that data on the impact of the environment on subsequent generations can be acquired quickly across the life-course. Table 1.2 summarises the renal outcomes in offspring exposed to the adverse maternal environments discussed in this section.

1.5.1 Animal models of maternal under-nutrition

1.5.1.1 Dietary Manipulation - Maternal calorie restriction

Maternal calorie restriction is used as a model for under-nutrition during pregnancy in humans and leads to LBW through IUGR. Calorie restriction has been performed in a range of animal species from sheep to rodents. Within these studies the extent of calorie restriction ranges from 20 – 70% of the normal recommended daily intake. These models demonstrate that reducing the amount of calories provided to females during pregnancy and lactation results in cardiometabolic abnormalities in the offspring including predisposition to weight gain, insulin insensitivity, increased BP (Woodall et al. 1996; Ozaki et al. 2001; George et al. 2012), accelerated ageing of the cardiovascular system (Kuo et al. 2017), vascular dysfunction (Nishina et al. 2003), as well as altered hypothalamic-pituitary-adrenal (HPA) axis activity (Ramírez-lópez et al. 2016) and liver function (George et al. 2012). The findings mirror those seen in humans from the Dutch famine cohort and therefore emphasise the efficacy of calorie restriction in animals for modelling maternal under-nutrition in humans.

Rodent models of under-nutrition also mirror the association between LBW and renal abnormalities in humans. Rat offspring exposed to 50% maternal calorie restriction throughout pregnancy had fewer glomeruli within their kidneys at birth and these glomeruli underwent hypertrophy in later life (Lucas et al. 1997; Almeida & Mandarim-De-Lacerda 2005). Glomerular hypertrophy is suggestive of a reduction in renal functional reserve. Consistently, these rats also had increased albuminuria, a decreased GFR and hypertension (Almeida & Mandarim-De-Lacerda 2005). These results suggest that a reduced nephron compliment at birth could be a contributing factor towards hypertension, as is suggested in humans.

Another factor linking renal programming to hypertension within the setting of maternal under-nutrition could be glucocorticoid handling. 50% calorie restriction in sheep from early to mid-gestation led to increased expression of the glucocorticoid receptor (GR) and decreased expression of 11β-Hydroxysteroid dehydrogenase type 2 (11β-HSD2) mRNA in the kidneys of newborn lambs (Whorwood et al. 2001). 11β-HSD2 de-activates circulating cortisol, suggesting that these lambs may have experienced excessive renal glucocorticoid levels. This is consistent with the high levels of renal angiotensin II receptor type 1 (ATR1) mRNA that was also reported in these lambs since glucocorticoids are known to promote ATR1 expression. An alteration in glucocorticoid sensitivity would be expected to interfere with fluid-electrolyte homeostasis and vascular tone, providing another plausible mechanism by which maternal under-nutrition may program offspring hypertension.

Furthermore renal disease, as evidenced by a decreased GFR and an increase in glomerulosclerosis and interstitial lesions, has also been observed in rats exposed to maternal calorie restriction by 18 months of age (Regina et al. 2001). This highlights that early changes in renal morphology may promote accelerated renal ageing. These studies reflect findings in humans and underscore the importance of the supply of nutrients during fetal life for both renal development and long term renal health.

1.5.1.2 Dietary manipulation - Maternal protein restriction

In addition to the total nutrient supply, the balance of nutrients supplied to the developing fetus is essential for appropriate fetal development. In particular, the availability of protein is a key determinant of fetal growth (Bertram & Hanson 2001). It has been demonstrated that maternal protein intake correlates positively with offspring birth weight independently of overall energy intake (Moore et al. 2004). Therefore, animal models of maternal low protein intake during pregnancy have been used to investigate the impacts of IUGR imposed by macronutrient deficiency on long-term offspring health.

The impacts of maternal protein restriction on offspring cardiovascular health and metabolism have been well studied. A maternal low protein diet during gestation has been demonstrated to influence offspring pancreatic development, reducing beta cell proliferation and leading to impaired insulin secretion in later life (Bertram & Hanson 2001). This provides a potential mechanistic link between low birth weight and later life diabetes in humans. Hypertension is also a well characterised outcome in offspring exposed to maternal protein restriction (Langley-Evans et al. 1996; McMullen & Langley-evans 2005) also reflecting findings in low birth weight humans. Additionally, there is evidence that the programming of hypertension may be sensitive to very modest alterations in maternal nutritional balance. Langley-Evans and colleagues investigated the effect of two different low protein diets which

differed in overall fat content, fatty acid composition, methionine content and the source of carbohydrate, and found that while both elicited a decrease in offspring growth, only one increased offspring blood pressure (Langley-evans 2000). This study emphasises the importance of the balance of other nutrients available to the growing fetus, in addition to protein, for later life offspring health.

Importantly, increases in offspring blood pressure following an early life low protein diet have been linked to renal development and function. Hypertension in rat offspring exposed to maternal protein restriction was associated with increased blood sodium concentrations (Manning & Vehaskari 2001). Accordingly, these offspring showed an upregulation of the $Na^+/K^+/2Cl^-$ cotransporter and Na^+/Cl cotransporter, proteins important for ion absorption in the ascending limb of the loop of Henle and in the distal tubule respectively (Manning et al. 2002). Consistently, an increase in thick ascending limb chloride transport has been observed in offspring from low protein mothers (Dagan et al. 2009). Imbalances in ion homeostasis within the kidneys of offspring exposed to a low protein diet may be interlinked with inappropriate glucocorticoid exposure during development. Mild maternal protein restriction increased mRNA and protein levels of GR and decreased levels of 11β-HDS2 mRNA in offspring kidneys. A concomitant increase in corticosteroid-responsive Na^+/K^+-ATPase α1- and β1-subunit mRNA was also observed, suggesting an alteration in offspring renal sodium absorption. Interestingly, these observations were seen before birth and persisted into adult life, implicating glucocorticoid hormone action as a potential programming factor linking poor fetal nutrition with sustained alterations in ion handling (Bertram et al. 2001)**.** These studies indicate that programmed impairments in ion homeostasis due to inadequate early life nutrition may be a contributing factor to later life hypertension. Conversely, it has also been shown that rats exposed to a low protein diet during gestation showed an increase in urine flow and sodium excretion by 4 weeks of age, and that this was correlated with hypertension (Alwasel & Ashton 2009). The increased sodium excretion was seen in these rats despite an increase in the expression of the $Na^+/K^+/2Cl^-$ cotransporter. The authors went on to show that low protein exposed rats showed an increase in salt and food intake, and suggested that this might be a compensatory mechanism to overcome programmed renal salt wasting (Alwasel et al. 2012). This suggests that the programming of hypertension by a maternal low protein diet could occur indirectly via altered renal sodium handling.

Consistent with observations of low birth weight in humans and with experimental studies of maternal calorie restriction, rats exposed to a maternal low protein diet have been shown to exhibit fewer nephrons and higher blood pressure (Vehaskari et al. 2001; Alwasel et al. 2010). Alterations in the RAS have been consistently seen along with a reduced nephron compliment and hypertension in low protein exposed offspring (Woods et al. 2001; Sahajpal & Ashton 2003; McMullen et al. 2004;

Vehaskari et al. 2004; Alwasel et al. 2010; Cooke et al. 2014), suggesting the RAS may be an important programming mechanism affecting the offspring renal and cardiovascular systems.

Notably, offspring with nephron deficit resulting from early life protein restriction have been seen to develop renal damage when challenged with a later life environmental insult. Sheep offspring exposed to a low protein maternal diet developed albuminuria only when they were fed an obesogenic diet (Lloyd et al. 2012). Moreover rats exposed to maternal protein restriction had a low nephron endowment and developed hypertension and renal hyperfiltration only when they were challenged with an infusion of advanced glycation end products (Zimanyi et al. 2006), suggesting a programmed susceptibility to diabetic nephropathy. These findings highlight that a poor early life environment may predispose offspring for adverse renal health but that renal disease may only develop when offspring are faced with a secondary challenge such as a poor postnatal environment.

1.5.1.3 Postnatal catch up growth

As mentioned previously, Eriksson and colleagues found that death rates from CVD were highest in men who were thin at birth but whose weight caught up to average or above average from 7 years (Eriksson et al. 1999). This observation led to the idea that the negative later life effects of a poor *in utero* nutritional environment may be exacerbated by an improved postnatal environment or secondary insult as described above. Animal models of "catch-up growth" have therefore been used to investigate the impacts of this phenomenon.

Rats exposed to a low protein diet *in utero* and then cross-fostered to normal protein fed dams in the lactation period showed LBW with accelerated growth during suckling (Jennings et al. 1999). This catch-up growth reduced longevity in these animals. Lifespan has also been demonstrated to be further reduced by exposure to a post-weaning cafeteria diet in mice who experienced catch up growth in early life. Consistently, mice exposed to a normal maternal protein diet during gestation and who were cross fostered to low protein dams during suckling have been observed to have increased longevity, and this longevity was protected even when these animals were challenged with a post-weaning cafeteria diet (Ozanne & Hales 2004). Furthermore, accelerated growth during postnatal life has also been associated with increased blood glucose levels in rats (Martin-Gronert et al. 2008). These findings mirror those seen in epidemiological studies.

It has been postulated that reduced longevity after early life catch up growth may occur due to accelerated ageing. Telomeres are known to shorten with age in most somatic tissues and it has been shown that rats exposed to *in utero* growth restriction and postnatal catch up growth display a shortening of telomeres in the kidney and pancreatic islets (Jennings et al. 1999; Tarry-Adkins et al. 2009). Notably, markers of cellular senescence, another hallmark of ageing, were also increased in the

pancreatic islets of “catch-up” offspring (Tarry-Adkins et al. 2009), whilst markers of DNA damage and oxidative stress were increased in the hearts of these offspring (Tarry-Adkins et al. 2013). These studies provide possible mechanisms linking the nature of early life growth with later life T2DM and CVD.

Mice exposed to catch-up growth in early life also show an age-dependent increase in albuminuria accompanied by alterations in genes related to oxidative stress and ageing within the kidney (Chen et al. 2010). Another study investigated the impact of catch up growth upon the offspring kidney by exposing rats to a low protein diet during gestation resulting in IUGR, and then over-feeding during the early postnatal period. Whilst blood pressure and renal function were unaffected in IUGR offspring despite showing a reduction in nephron number, those exposed to IUGR with postnatal over-feeding displayed hypertension, glomerulosclerosis and a reduction in GFR (Boubred et al. 2009). Again, this study highlights the notion that a second insult in addition to a poor *in utero* environment may be necessary for a poor renal phenotype in later life. Together, these studies reiterate renal health as an important factor in cardiovascular health and mirror epidemiological findings.

1.5.2 Animal models of maternal hormone modulation

1.5.2.1 Maternal diabetes

As mentioned above, diabetes during pregnancy is becoming a common occurrence during pregnancy and is known to have adverse consequences for both mother and child in the short and long term. Diabetes can be induced in rodent models by streptozocin (STZ) administration which destroys the pancreatic beta cells, impairing insulin release and rendering the animal diabetic. This is a severe method of inducing diabetes and can lead to weight loss and hyperglycaemia within 48 hours (Deeds et al. 2013). Although STZ administration alters maternal glucose homeostasis, it’s important to note that it is not reflective of diabetes in human pregnancy (this is discussed in chapter 4). The extent of STZ administration may lead to differing offspring outcomes. Whereas a mild STZ challenge has been shown to increase offspring birth weight (Merzouk et al. 2000), mirroring findings in humans exposed to gestational diabetes, a harsher challenge has been shown to decrease offspring birth weight (Hokke et al. 2013). Like other adverse early life environments, exposure to maternal diabetes induced by STZ has been shown to increase blood pressure (Wichi et al. 2005), and impair the metabolism (Merzouk et al. 2000) of exposed offspring.

Like studies of under-nutrition, maternal STZ induced diabetes appears to impair offspring renal development and function and these abnormalities are often observed with hypertension (see Table 1.2). These observations will be discussed in chapters 4, 5 and 6.

1.5.2.2 Maternal glucocorticoids

Glucocorticoids and other hormones have a key role in regulating development *in utero* and therefore may significantly influence the progression of adult disease. Throughout the majority of fetal life, the maternal placental enzyme 11β-HSD converts active cortisol to inactive cortisone, thereby protecting the developing fetus from glucocorticoid over-exposure. However during late gestation, fetal glucocorticoid levels rise to mature the fetal tissues in preparation for extra-uterine life. The ability of synthetic glucocorticoids to mature fetal tissues, particularly the lung, underpins their widespread antenatal use to improve fetal outcomes in threatened preterm delivery (Seckl & Holmes 2007). However, placental 11β-HSD is known to be downregulated in human pregnancies complicated with IUGR (Nuyt 2008), and in rats exposed to a low protein diet (Langley-Evans et al. 1996). Additionally, a variety of maternal conditions associated with growth restriction including stress, nutrient restriction and placental insufficiency are known to increase fetal glucocorticoid levels (Seckl & Holmes 2007). Glucocorticoids therefore provide a plausible link between adverse maternal conditions and poor offspring outcomes.

Models of glucocorticoid overexposure either utilise betamethasone and dexamethasone, exogenous glucocorticoids which are not catalysed by 11β-HSD, or endogenous glucocorticoids coupled with the inhibition of 11β-HSD. Both methods of glucocorticoid overexposure lead to growth restriction in the offspring. Since growth restriction promotes adult disease in humans, this supports the hypothesis that glucocorticoids may be involved in the early life programming of adult disease (Seckl 2001).

Animal models have provided evidence that inappropriate antenatal exposure to glucocorticoids leads to growth restriction in the offspring, produces permanent hypertension, hyperglycaemia, hyperinsulinaemia, altered behaviour and neuroendocrine responses throughout the lifespan [reviewed in (Seckl 2001)]. Alteration of the HPA axis negative feedback set point in offspring over-exposed to glucocorticoid in early life leads to chronic high levels of circulating glucocorticoid. This, together with the adverse programming of glucocorticoid receptors in organs across the body, can alter glucocorticoid action and promote disease in adulthood (Seckl 2001).

Interestingly, sheep programmed to become hypertensive by exposure to dexamethasone very early on in gestation had fewer nephrons at 7 years of age. Their glomeruli were also larger, suggesting individual glomerular compensation to maintain GFR (Wintour et al. 2003). This study suggests that hypertension resulting from early life glucocorticoid exposure may result in part from the

programming of low nephron endowment and mirrors other models of a poor intrauterine environment. However, pregnant mice infused with dexamethasone during mid-gestation also had offspring with fewer nephrons at 20 weeks of age (Dickinson et al. 2007), but these offspring showed normal basal blood pressure levels relative to controls. These studies suggest that the timing of glucocorticoid excess is important for programming outcomes.

There is also evidence that early life glucocorticoid overexposure programs other factors in the kidney which might contribute to later life hypertension. Glucocorticoid levels rise at the time of birth and this coincides with the kidneys ability to absorb sodium. It has been demonstrated that sheep administered with cortisol during late gestation show a rise in Na^+/K^+-ATPase that is equivalent to newborn lambs (Petershack et al. 1999), demonstrating a direct link between glucocorticoid levels and ion handling in the kidney. Accordingly, rats exposed to dexamethasone in mid to late gestation had a 50% increase in Na^+/H^+ exchanger activity in the proximal tubules and an increase in sodium-hydrogen exchanger 3 protein abundance on brush-border membrane vesicles. There was a corresponding increase in proximal convoluted tubule volume absorption and this was associated with elevated blood pressure by 7-8 weeks of age (Dagan et al. 2007).

The renal RAS has also been shown to be altered by fetal glucocorticoid excess and this is another potential factor linking adverse early life conditions to both renal and cardiac abnormalities. By late gestation, sheep fetuses exposed to dexamethasone during early gestation showed increases in renal ATR1 and angiotensin II receptor type 2 (ATR2) together with an altered GFR and urine flow in response to Ang II infusion (Moritz et al. 2002). This emphasises that renal function can be impaired by a programmed alteration in the renal RAS. Chronic alterations in renal function may promote disease in later life. Accordingly, male adult sheep exposed to antenatal betamethasone showed a heightened susceptibility to renal oxidative stress (Bi et al. 2014), demonstrating that glucocorticoid excess may prime the kidney for accelerated ageing and damage during adulthood.

1.5.3 Animal models of maternal over-nutrition

Obesity or maternal over-nutrition is becoming extremely common in women of reproductive age and is known to lead to immediate adverse outcomes for both mother and child as discussed above. Animal models have been set up to further explore the impact of a high fat and/or sugar diet as well as the impact of maternal obesity on the later life health of exposed offspring.

1.5.3.1 Maternal High fat diets

Within western society the typical diet is very high in saturated fat. Therefore, animal models where the mother is fed a high fat diet throughout gestation and lactation have been utilised to explore the mechanisms behind adverse programming in response to maternal over-nutrition. Within these models the percentage of calories attained from fat ranges from 18 – 60% in mice, rats and non-human primates [reviewed in (L. Williams et al. 2014)].

The impacts of a maternal high fat diet on offspring outcomes in rodents have been fairly variable, with placental alterations leading to both high and low birth weights in the offspring. Low birth weight may result from placental dysfunction caused by an adverse diet whereas high birth weight is associated with an increase in placental nutrient transport. Differential outcomes may arise due to differences in dietary composition, including the amount and type of fat used as well as other nutrients, the timing of exposure and the species utilised. Additionally, the presence of confounding factors in the mother such as obesity and insulin resistance may also lead to differential outcomes in the offspring (L. Williams et al. 2014). Regardless, maternal high fat diets have been shown to lead to features of the metabolic syndrome in exposed offspring including insulin resistance, glucose intolerance, dyslipidaemia, increased liver mass and triglyceride content, hepatic steatosis, increased visceral fat mass, adipocyte hypertrophy and high blood pressure [reviewed in (L. Williams et al. 2014)]. High blood pressure may be a cause or consequence of reduced endothelium relaxation and increased arterial stiffness (Franklin 2005), features which have also been observed in adult offspring exposed to a maternal high fat diet (Armitage et al. 2005). One limitation of utilising high fat diets to study the effects of maternal over-nutrition is that this diet is not translatable to the human condition. This is because a high fat diet alone is not palatable, especially in rodents, and human over-nutrition is characterised by increased calorie intake.

Non-human primates provide a model of pregnancy that is more similar to humans in terms of development and the fact that mothers bear singleton or twin pregnancies. As such, they have been extensively utilised to study the impacts of a maternal high fat diet on offspring health. Exposure of non-human primates to a maternal high fat diet has been shown to lead to a 3-fold increase in fetal liver triglycerides along with histologic changes indicative of non-alcoholic fatty liver disease. These changes were observed together with epigenetic alterations of key genes involved in energy homeostasis in the liver (Aagaard-Tillery et al. 2008). Importantly, these alterations could be reversed by a change in maternal diet which did not result in weight loss in the mother (McCurdy et al. 2009; Suter et al. 2012). These studies highlight the importance of nutrient availability and not obesity *per se* in the programming of an adverse offspring liver phenotype. Non-human primates also seem to be susceptible to vascular defects in response to a maternal high fat diet. Intima thickness, a measure of

atherosclerosis was increased in offspring exposed either to a maternal or post-weaning high fat diet. Importantly, offspring exposed to both conditions displayed the most pronounced atherosclerotic profile highlighting an additive effect of a maternal and later life high fat diet on vascular damage (Fan et al. 2013).

Like other animal models of adverse early life environments, a maternal high fat diet has been demonstrated to lead to alterations in the offspring kidney (see Table 1.2). These will be discussed in chapters 4-6.

1.5.3.2 Maternal obesogenic diet (high fat and sugar western diet)

Obesity in women of reproductive age is increasing at an alarming rate across the globe, with adverse consequences for both mother and child as discussed above. The typical western diet resulting in obesity is high in both fats and simple sugars. Therefore, models utilising a palatable "energy dense" or "western" diet rich in both fats and simple sugars before and throughout gestation and lactation have been employed to better replicate the human condition and investigate the mechanisms leading to adverse offspring outcomes. A diet high in fats and sugars is particularly useful for modelling human obesity in rodents, since rodents are less capable of negatively regulating their energy intake when fats and sugars are combined. These diets are therefore termed "palatable" to reflect this advantage.

Maternal obesity induced by feeding a palatable obesogenic diet for 6 weeks prior to mating, throughout gestation and lactation was shown to induce hyperphagia and increase adiposity, insulin and glucose levels in offspring (Samuelsson et al. 2008). Offspring were also hypertensive by 3 months of age. Although hypertension is often promoted by an increase in body weight, Samuelsson and colleagues demonstrated that hypertension develops in offspring exposed to maternal obesity before a significant increase in adiposity (Samuelsson et al. 2009). This was associated with sympathoexcitatory hyper-responsiveness involving the brain, vasculature and kidney. Furthermore, our laboratory demonstrated that male offspring of obese dams displayed cardiac hypertrophy (Blackmore et al. 2015) associated with hyperinsulinemia and increased stimulation of the mitogen-activated protein kinase pathway (Fernandez-twinn et al. 2012). These offspring also showed systolic and diastolic dysfunction and sympathetic dominance by 12 weeks of age (Blackmore et al. 2015). Again this phenotype was observed before the onset of offspring adiposity, suggesting that exposure to early life obesity directly primes the offspring for poor cardiovascular health.

As mentioned above, in models of maternal over-nutrition, it can be difficult to tease apart the programming impacts of dietary intake and obesity *per se*. Frihauf and colleagues set up a model that used genetic strains of mice that were either resistant or susceptible to diet induced obesity. Females were then fed either a control or western diet throughout pregnancy and lactation. Offspring exposed

to a maternal western diet showed increases in adiposity despite mothers showing a normal energy intake, body weight and pregnancy weight gain (Frihauf et al. 2016). Conversely, obesity in mothers despite exposure to a control diet also appeared to induce metabolic perturbations in mice. Importantly, the combination of maternal obesity and a western diet produced an additive effect on offspring insulin and leptin levels. This emphasises that both obesity and diet composition are important and independent programing factors that influence the offspring metabolism.

There is evidence that a maternal obesogenic diet impairs offspring renal function and health in both sexes (Table 1.2). This will be discussed in chapters 4-6. Unlike other models of maternal adverse conditions in pregnancy, the programming effects leading to poor renal health in later life remain unexplored within the setting of maternal obesity. Illuminating such programing mechanisms might shed light on the aetiology of renal and cardiovascular dysfunction in offspring exposed to maternal obesity.

Table 1.2 *Animal models of adverse maternal environments and renal outcomes in offspring*

Early life condition	Species	Phenotype	Renal phenotype	Reference
Calorie Restriction				
50% throughout gestation	Rat	↓ birth weight	↓ relative renal weight ↓ nephrons ↑ glomerular hypertrophy ↔ tubular function	(Lucas et al. 1997)
50% until mid gestation	Sheep	↔ birth weight ↑ placenta/ body weight	↑ renal weight ↑ renal GR ↓ renal 11βHSD2 mRNA ↑ ATR1 mRNA	(Whorwood et al. 2001)
50% throughout gestation	Rat	↓ birth weight	↓ renal/ body weight ↓ nephrons ↑ glomerular hypertrophy ↓ GFR ↑ RPF ↑ Glomerulosclerosis	(Regina et al. 2001)
50% throughout gestation	Rat	↓ birth weight ↑ blood pressure	↓ renal/ body weight ↓ nephrons ↑ glomerular hypertrophy ↓ GFR ↑ albuminuria	(Almeida & Mandarim-De-Lacerda 2005)
Protein Restriction				
6% from mid gestation	Rat	↓ birth weight ↑ blood pressure	↑ tubular sodium reabsorption	(Manning et al. 2002)
6% from mid gestation	Rat	↑ blood pressure	↑ tubular sodium reabsorption	(Dagan et al. 2009)
9% throughout gestation	Rat	↓ birth weight ↑ blood pressure	↑ GR ↓ 11βHSD2 mRNA ↑ Na^+/K^+-ATPase	(Bertram et al. 2001)

6% from mid gestation	Rat	↓ birth weight ↑ blood pressure ↓ plasma renin ↑ plasma aldosterone	↓ nephrons ↑ renal apoptosis	(Vehaskari et al. 2001)
8% throughout gestation and lactation	Rat	↓ weight (E19 + PN21)	↑ renal renin at E19 ↑ renal ACE at E19 ↑ ATR1 at PN21	(Cooke et al. 2014)
8.5% throughout gestation	Rat	↓ birth weight ↑ blood pressure	↓ renal/body weight ↓ nephrons ↑ glomerular hypertrophy ↓ renal renin ↓ renal Ang II ↑ GFR	(Woods et al. 2001)
9% throughout gestation	Rat	↓ body weight ↑ blood pressure	↓ nephrons ↑ renal vascular resistance ↑ ATR1 ↑ response to Ang II	(Sahajpal & Ashton 2003)
9% throughout gestation	Rat	↑ blood pressure	↓ nephrons ↓ ATR2 ↑ response to Ang II	(McMullen et al. 2004)
6% throughout gestation	Rat	↓ birth weight ↑ blood pressure	↓ ATR1 & ATR2 at PN1 ↑ ATR1 & ATR2 at PN28	(Matti Vehaskari et al. 2004)
9% throughout gestation	Rat	↓ birth weight	↓ nephrons ↓ ATR1 and ATR2 between E19 and PN10	(Alwasel et al. 2010)

9% throughout gestation	Rat	↑ blood pressure	↔ GFR ↑ urine flow ↑ sodium excretion ↑ $Na^+/K^+/2Cl^-$ cotransporter ↓ Na+/K+ ATPase α1 subunit in inner medulla	(Alwasel & Ashton 2009)
8% throughout gestation	Sheep		↑ apoptosis in nephrogenesis ↓ angiogenesis ↓ capillary density ↓ nephrons ↑ glomerular hypertrophy	(Lloyd et al. 2012)
8.7% throughout gestation and lactation	Rat	↓ body weight	↓ GFR ↑ collagen associated AGEs	(Zimanyi et al. 2006)
<u>Postnatal Catch Up Growth</u>				
8% protein during gestation, 20% protein during lactation	Rat	↑ body weight at weaning	↑ albuminuria Changes in gene expression relating to reduced antioxidant capacity and protein homeostasis	(Chen et al. 2010)
9% protein during gestation, reduced litter size and 22% protein during lactation	Rat	↓ birth weight ↑ central adiposity in adulthood ↑ blood pressure	↓ renal/body weight ↓ nephron number ↑ glomerular hypertrophy ↑ glomerulosclerosis ↓ creatinine clearance ↑ proteinuria	(Boubred et al. 2009)
<u>Diabetes</u>				
STZ for 3 consecutive days starting E6.5 at doses 100, 100, and 80mg/kg of body7 weight	Mouse	↓ fetal weight	↓ ureteric tip number ↓ ureteric tree length ↓ nephron number ↑ kidney malformations	(Hokke et al. 2013)

STZ 150mg/kg of body weight at E13	Mouse	↓ body weight	↓ kidney weight ↑ kidney weight/body weight ↓ nephron number ↓ glomerular volume ↑ apoptosis in developing nephrons ↑ intra-renal angiotensinogen and renin mRNA	(Tran et al. 2008)
35mg/kg of body weight at E0	Rat	↑ body weight with age ↑ blood pressure ↓ plasma renin activity	↓ nephron number ↑ proteinuria ↓ GFR ↑ ion channels expression in the cortex	(Nehiri et al. 2008)
50mg/kg 10 days prior to mating	Rat	↔ body weight ↑ blood pressure	↔ nephron number ↑ glomerular hypertrophy ↓ GFR	(Magaton et al. 2007)
60mg/kg prior to mating	Rat	↔ body weight ↑ blood pressure	↑ glomerular area ↑ immune cell infiltration ↑ renal vascular resistance ↓ renal plasma flow ↓ GFR	(Rocco et al. 2008)
50mg/kg 10 days prior to mating	Rat	↑ body weight with age ↑ blood pressure ↓ endothelium dependent vasodilatation	↔ nephron number ↑ glomerular hypertrophy ↓ renal plasma flow ↓ GFR	(Rocha et al. 2005)

150mg/kg at E13	Mouse	↓ body weight ↑ blood pressure ↓ glucose tolerance	↓ kidney weight ↑ albuminuria ↑ glomerulosclerosis ↑ angiotensinogen, ATR1, ACE mRNA	(Chen et al. 2010)
25mg/kg at E1	Rat	↑ birth weight ↑ blood pressure ↓ glucose tolerance	↔ nephron number ↑ creatinine clearance ↑ urinary NAG	(Yan et al. 2014)
150mg/kg at E13	Mouse	↓ birth weight ↓ body weight ↑ blood pressure ↑ plasma lipids ↓ glucose tolerance	↑ albuminuria ↑ GFR ↑ glomerulosclerosis ↑ tubulointerstitial fibrosis	(Aliou et al. 2016)
<u>Glucocorticoids</u>				
Dexamethasone (0.48 mg/h for 48h) in early gestation	Sheep	↔ birth weight ↑ blood pressure	↓ nephron number ↑ glomerular volume ↔ glomerulosclerosis ↑ proximal tubule area ↑ collagens in tubular interstitium	(Wintour et al. 2003)
Dexamethasone (125 µg/kg for 60h) at mid-gestation	Mouse	↔ birth weight ↔ blood pressure	↓ nephron number ↑ genes inhibiting ureteric branching in fetal kidneys	(Dickinson et al. 2007)
48h intraperitoneal infusion cortisol in late gestation	Sheep		↑ Na^+/K^+-ATPase activity and protein expression in fetus	(Petershack et al. 1999)

Dexamethasone (0.2 mg/kg) daily for 4 days mid -gestation	Rat	↓ birth weight ↑ blood pressure	↑ proximal tubule volume absorption ↑ Na^+/H^+ exchanger activity in proximal tubule	(Dagan et al. 2007)
Dexamethasone (0.48 mg/h for 48h) in early gestation	Sheep	↔ fetal weight	↑ renal angiotensinogen, ATR1 and ATR2 mRNA ↑ urine flow and GFR in response to Ang II infusion	(Moritz et al. 2002)
Betamethasone (two 0.17-mg/kg intra-muscular injections to mothers) at mid-gestation	Sheep		↑ 8-isoprostane and protein excretion in response to Ang II infusion in male offspring only	(Bi et al. 2014)
<u>High fat diet</u>				
20% lard throughout pregnancy and lactation	Rats	↔ birth weight ↔ body weight ↑ blood pressure ↑ aortic stiffness ↓ endothelium dependent relaxation	↔ kidney weight ↔ nephron number ↔ glomerular volume ↓ renin activity ↓ Na^+/K^+-ATPase activity	(Armitage et al. 2005)
21% fat throughout gestation and lactation	Mouse	↔ birth weight	↑ nephron number ↔ renal morphology and function	(Hokke et al. 2016)
13% fat throughout gestation and lactation	Rabbit	↑ body weight ↑ blood pressure ↑ heart rate	↑ renal sympathetic nerve activity ↑ renal sympathetic nerve activity in response to stress and central leptin infusion	(Prior et al. 2014)

43% fat throughout gestation and lactation	Mouse	↔ body weight	↑ fibrosis ↑ oxidative stress ↑ inflammation	(Glastras, Tsang, et al. 2016)
<u>Obesogenic diet</u>				
45% high fat diet with water containing 0.1 g/ml fructose throughout gestation and lactation	Rat	↔ birth weight ↔ body weight ↔ blood pressure	↔ GFR ↑ glomerulosclerosis	(Jackson et al. 2012)
45% high fat diet with water containing 0.1 g/ml fructose throughout gestation and lactation	Rat (female offspring)	↔ birth weight ↔ body weight ↔ blood pressure	↔ GFR ↓ nestin expression ↑ TGF-β expression	(Flynn et al. 2013)

1.6 The process of renal development

In order to understand how the kidney can be programmed during early life in response to different environments, an understanding of the process of renal development is required.

During fetal development in mammals, 3 separate kidney structures are formed; the pronephros, mesonephros and metanephros (mature excretory organ). The pronephros develops very early on in gestation, is rudimentary and does not function. As the pronephros regresses, the mesonephros begins to develop. The mesonephros comprises pairs of primitive glomeruli which filter into mesonephric tubules and into the mesonephric duct. It is thought to function for a short time in early gestation. The metanephros, the permanent kidney, begins to develop in early gestation as the mesonephros starts to regress. Figure 1.3 describes these 3 renal kidney structures. The rest of this section will describe the process of metanephros development.

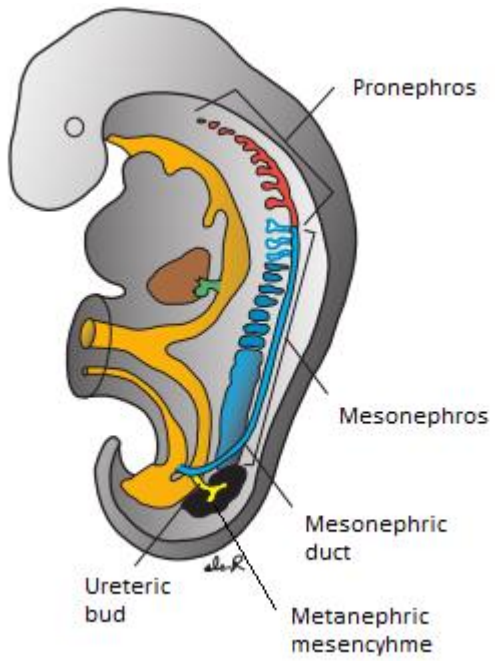


Figure 1.3. **Kidney structures within an early mammalian embryo.** Red = Pronephros. Blue = Mesonephros. Black = metanephric mesenchyme. The metanephric mesenchyme together with the ureteric bud comprise the metanephros. Figure adapted from (Sadler 2011).

1.6.1 Renal development in the mouse vs human

Renal development occurs over a relatively long time period. This might be one of the reasons it appears to be so susceptible to adverse early life environments. If we are to understand the effects of the early life environment on the kidney, we need to understand the timeframe of renal development in humans relative to models such as the mouse. Figure 1.4 shows a simplistic timeline of the renal developmental events in humans and mice.

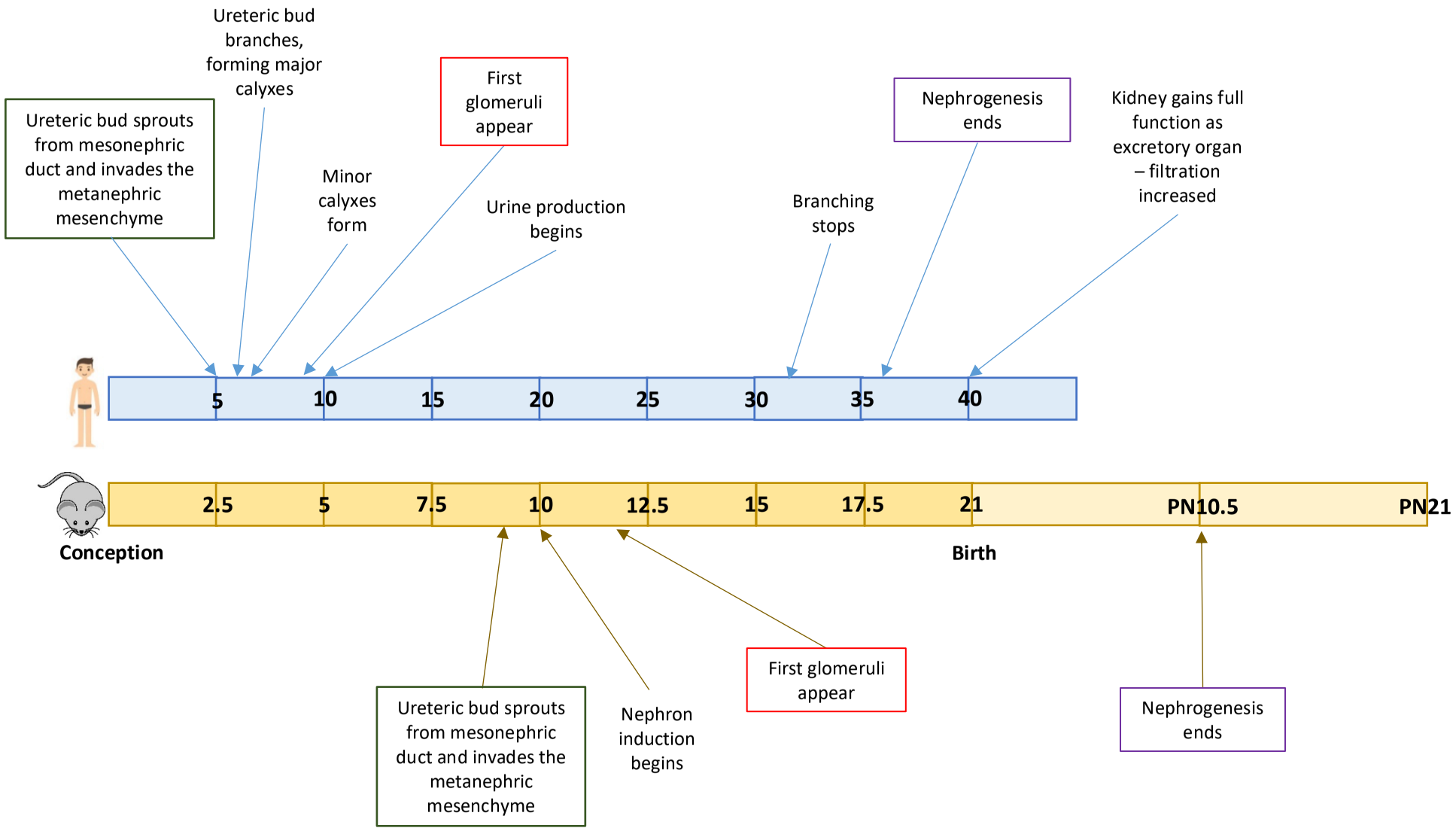


Figure 1.4. **Timeline of human vs. mouse metanephric kidney development (until nephrogenesis completion).** It should be noted that both the human and rodent kidney continue to develop following the completion of nephrogenesis. GFR and active solute recovery increase postnatally.

1.6.2 The metanephric kidney

The mammalian metanephric kidney has two portions derived from separate tissues. The collecting system originates from the ureteric bud whereas the excretory system develops from the metanephric mesenchyme.

1.6.2.1 The collecting system

The ureteric bud develops at first from the mesonephric duct. The bud invades the metanephric mesenchyme and bifurcates. Each branch end acquires a cap of mesenchymal cells (cap mesenchyme). These two initial branches will form the renal pelvis and ureter (Figure 1.5.A). After this initial bifurcation, the ureteric bud goes through 4 more rounds of branching. The resulting branches fuse to form the major calyces (Figure 1.5.B), through which urine will eventually pass before moving to the renal pelvis and ureter. Another generation of branches also fuse to form the minor calyces (Figure 1.5.D), through which urine will pass before reaching the major calyces. Branching continues until the collecting tubules of the mature kidney are formed (Figure 1.5.D) (Sadler 2011).

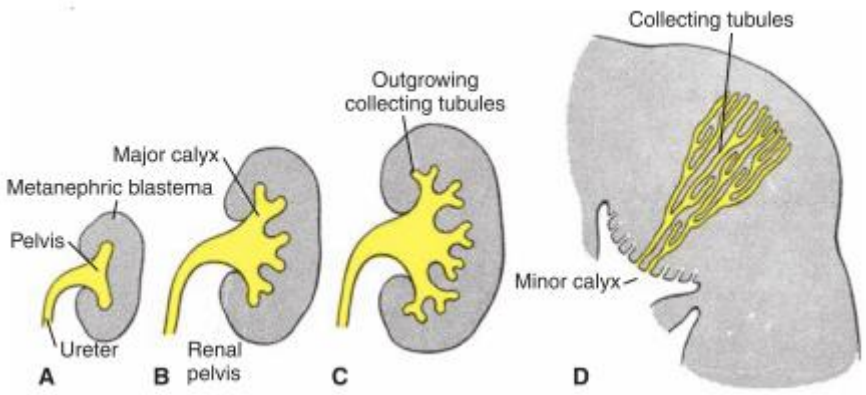


Figure 1.5. **Development of the collecting system of the metanephric kidney in the mammalian fetus. A)** The ureteric bud branches. This structure will form the renal pelvis. **B)** The next round of branches fuse to form major calyxes. **C)** Branching continues. These structures will form the minor calyxes. **D)** Branching results in collecting tubules. These join the minor calyxes in pyramid structures. Figure taken from (Sadler 2011)

1.6.2.2 The excretory system

Throughout branching morphogenesis, each branch tip acquires its own cap mesenchyme (Figure 1.6.A). Cap mesenchyme is induced by growth factors excreted from the ureteric bud to form clusters of migratory cells which undergo the mesenchyme to epithelial transition (Figure 1.6.B). These structures develop from vesicles, to comma shaped, to S shaped bodies (Figure 1.6.C), and eventually nephrons with proximal and distal polarity. Development of the glomerulus starts at the S shaped body stage, when precursor cells at the most proximal end begin to express proteins specific to podocytes (Dressler 2006). The distal end of the developing nephron joins to the ureteric bud it originated from, forming a continuous collecting duct (Figure 1.6.C & D). The proximal end expands and forms a Bowman's capsule (Figure 1.6.C & D), into which the glomerulus will filter (Figure 1.6.E & F). The final stage of development of the nephron involves the elongation of the tubules, forming a mature proximal convoluted tubule, loop of Henle and distal convoluted tubule (Figure 1.6.F). The formation of new nephrons ends at 36 weeks gestation in humans (Reidy & Rosenblum 2009) and 1-2 weeks following birth in mice (Yosypiv 2014)(Figure 1.4). Following the end of nephrogenesis, the kidney continues to grow in size. This is due to further lengthening of the renal tubules and growth of the interstitial tissue (Sadler 2011).

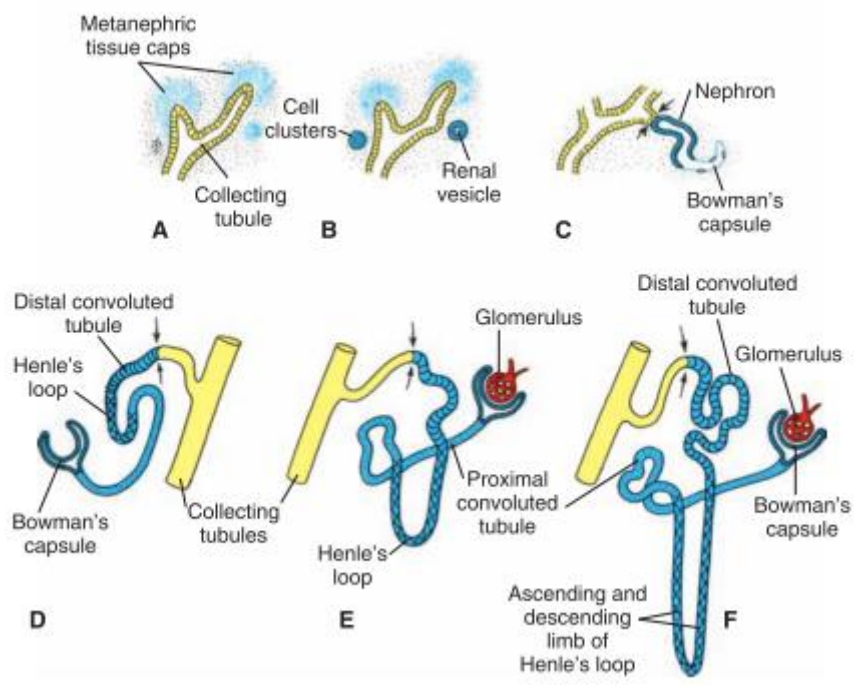


Figure 1.6. **Nephrogenesis in the mammalian kidney.** Yellow structures show the collecting tubules, derived from the ureteric bud. Blue structures show the developing nephron, derived from metanephric mesenchyme. Arrows indicate where the nephron (excretory unit) join the collecting ducts (collecting unit). Figure taken from (Sadler 2011).

1.6.2.3 Establishment of the vasculature and nerves

The renal vasculature has two origins. The renal artery and its larger vessels develop from a branch of the aorta, whereas capillaries develop from cells within the metanephric mesenchyme. Endothelial cells of the capillaries invade the S shaped body of the developing nephron (Yosypiv 2014). Paracrine signalling from developing podocytes ensures developing endothelial cells migrate towards the podocytes. The meeting of these two cell types, along with the development of mesangial cells and the epithelial cells of the Bowman's capsule, gives rise to the mature glomerulus (Schell et al. 2014). Developing endothelial cells are also attracted to the loop of Henle, forming the vasa recta (Davies 2013).

The renal nerves develop in close association with the vasculature (Kett & Denton 2011). The timing and nature of development of the efferent and afferent nerves differs. It has been shown in the rat that afferent nerves originate near the ureter and vessels entering the kidney and extend to the renal pelvis, the corticomedullary connective tissue and vasculature by birth. Conversely, efferent nerves are confined to the interlobular arteries and afferent arterioles of the juxtamedullary nephrons at birth. After birth, the efferent nerves grow rapidly and extend to the entire renal vascular network, including glomeruli within the cortex, by 21 days after birth (Liu & Barajas 1993).

1.6.2.4 Molecular control of kidney development

The development of the metanephric kidney relies on signalling between the metanephric mesenchyme and ureteric bud. A number of genes are known to play a key role in this process.

Ureteric bud outgrowth is known to be controlled by glial derived neurotrophic factor (*Gdnf*) expressed in the metanephric mesenchyme. GDNF binds to the receptor tyrosine-protein kinase receptor (RET) on the ureteric bud, promoting bud outgrowth towards the *Gdnf* signal (Dressler 2006; Patel & Dressler 2013). A number of genes control this pathway upstream including wilms tumour 1 (*Wt1*), homeobox (*Hox*) genes, paired box 2 (*Pax2*) and LIM homeobox 1 (*Lim1*) (Dressler 2006; Yosypiv 2012). Interestingly, the renin angiotensin system has also been shown to play a role in the maintenance of *Pax2* in the metanephric mesenchyme, thereby promoting ureteric bud morphogenesis (Yosypiv 2012). It is also known that bone morphogenetic protein 4 (*Bmp4*) expressed in the mesenchymal cells surrounding the nephric duct inhibits *Gdnf* signalling, thereby preventing excessive ureteric branching (Patel & Dressler 2013).

The induction of the metanephric mesenchyme leads to aggregation of mesenchymal cells around the ureteric bud (the cap mesenchyme). This induction is known to be controlled by Wnt family member 9b (*Wnt9b*) (Patel & Dressler 2013). The maintenance of cap mesenchyme is in then promoted by T

cell leukemia homeobox 1 (*Hox11*), EYA Transcriptional Coactivator and Phosphatase 1 (*Eya1*) and *Pax2* leading to the expression of sine oculis-related homeobox 2 (*Six2*), *Gdnf* and *Wt1* (Davies 2013). Nephron development requires cap mesenchymal cells to undergo condensation and the mesenchymal to epithelial cell transition. Wnt family member 4 (*Wnt4*) has been shown to be important for this condensation leading to renal vesicles with cell polarity (Dressler 2006; Patel & Dressler 2013). Renal vesicles then develop into S-shaped bodies. It has been demonstrated that Notch signalling is important for the formation of proximal-distal polarity within S-shaped bodies. Specifically, *Notch2* is integral for the formation of proximal cell types including proximal tubule cells and glomeruli (Patel & Dressler 2013).

1.6.3 Establishment of renal functionality

Functionality is acquired fairly slowly in the developing kidney. In the fetus, fluid and electrolyte balance and waste excretion are regulated by the placenta, such that the functioning kidney is not needed until after birth. The gain in renal function involves 4 main processes, the development of; glomerular filtration, selective solute recovery, active transport and hormone production (Davies 2013).

1.6.3.1 Glomerular filtration

The individual filtration rate of a nephron increases with its maturity. The overall glomerular filtration rate increases with fetal age, the number of glomeruli and blood pressure (Davies 2013). Overall, glomerular filtration is low during fetal life. Rates increase rapidly following birth due to a shift in demand. This increase is facilitated by increases in arterial and renal ultrafiltration pressure, and decreases in renal vascular resistance (Kett & Denton 2011). In the rat, renal blood flow and glomerular filtration rates are very low at birth, increase slowly up to 30 days of age and then increase rapidly to adult levels (Forty & Ashton 2012).

1.6.3.2 Solute recovery and active transport

Solute recovery is also acquired slowly in the fetal kidney. In the mouse, active anion recovery has been shown to be present at E13.5 (Davies 2013). However, like glomerular filtration, tubule maturation continues rapidly after birth due to an increase in functional demand and this facilitates an increase in solute recovery. The proximal tubules undergo folding of the apical membranes to increase the surface area for microvilli for reabsorption, and increased basal folding and mitochondria number for ATP generation to drive Na^+/K^+-ATPase (Kett & Denton 2011). It is thought that full

maturation of sodium and water reabsorption in the tubules of the rodent kidney takes up to 6 weeks after birth (Kett & Denton 2011). There is evidence that the urotensin system may be important for water and sodium balance before full maturity is reached; it has been shown that activation of the urotensin system increases renal blood flow, glomerular filtration, diuresis, natriuresis and sodium excretion in 4 week old rats (Forty & Ashton 2012).

1.6.4.3 Hormone production

The adult kidney has an important role in producing renin, erythropoietin and calcitriol (the active form of vitamin D). Hormone signalling begins in fetal life and is important for renal development. The juxtaglomerular apparatus is functional in the fetus and releases renin, activating the RAS (Davies 2013). Mice lacking a functional RAS during fetal life show poor ureteric bud branching and a lower nephron endowment (Yosypiv 2014). Erythropoietin is an important hormone responsible for erythropoiesis. In the fetus, erythropoietin production occurs in the liver. In the mouse, it has been shown that the switch from liver to renal production is completed around 2 weeks after birth (Pan et al. 2011).

1.7 The role of epigenetics in renal programming

Early life environmental perturbations, such as maternal under or over-nutrition, can lead to changes in placental function and ultimately the environment that the growing fetus experiences. Environmental cues can stimulate epigenetic modifications in genes, causing permanent changes to growth and later life health. The term “epigenetics” describes heritable modifications to the DNA that do not include changes to the DNA sequence itself (Egger et al. 2004). The best understood epigenetic mechanisms are DNA methylation of cytosine residues within CpG dinucleotides and methylation/acetylation of DNA packaging histone proteins. Other epigenetic mechanisms include non-coding or micro RNA’s (miRNAs) which degrade mRNA or block its translation and so act as post-transcriptional gene regulators (Pinnock & Ozanne 2016). During fetal development and early life, epigenetic signatures are laid down which persist throughout the life course. Importantly, the environment experienced at this time can influence epigenetic mechanisms. Generally, it has been demonstrated that offspring with IUGR have altered epigenetic marks (Ritz et al. 2011), suggesting early life insults may influence long term health through epigenetic mechanisms.

Epigenetic modifications have been shown to be involved in the pathogenesis of CKD. A hallmark of CKD is proteinuria. The slit diaphragm of the podocyte is integral to controlling glomerular filtration and preventing proteinuria. Recently it has been demonstrated in a mouse model that the RAS is involved in the epigenetic regulation of nephrin (a key component of the slit diaphragm). It was shown that Angiotensin II leads to the downregulation of Kruppel-like factor 4, allowing DNA methyltransferase 1 to bind to the nephrin promoter and leading to CpG hypermethylation. This reduces transcription of the nephrin gene and impairs the integrity of the glomerular filtration barrier (Feliers 2015). It has also been observed that kidney fibrosis, another hallmark of CKD, is stimulated by the hypermethylation of the *Rasal1* promoter, and that bone morphogenic protein 7 (BMP7) prevents this fibrosis by normalising the hypermethylation of *Rasa1* (Tampe et al. 2014). Multiple miRNAs have also been identified in the pathogenesis of renal fibrosis. miR-192 has been shown to be upregulated in a mouse model of unilateral uretal obstruction and a rat model of kidney disease associated with the activation of TGF-β (Wing et al. 2013). Furthermore, knock down of miR-192 was shown to prevent TGF- β mediated collagen expression (Wing et al. 2013). miR-29 has also been shown to be anti-fibrotic in a mouse model of obstructive nephropathy; miR-29 was shown to be reduced in mice with fibrosis, whilst Smad3 knock out mice had increased miR-29 and were protected from renal fibrosis (Wing et al. 2013). Importantly, several miRNAs have been associated with CKD in humans. Renal biopsy specimens from patients with hypertensive nephrosclerosis showed increased levels of miR-200a, miR-200b, miR-141, miR-429, miR-205, and miR-192 expression, and expression levels were correlated with the severity of disease (Wing et al. 2013).

The evidence that epigenetic modifications can arise due to early life perturbations, together with the fact that epigenetic modifications are involved in the pathogenesis of CKD, emphasises the potential involvement of the early life environment in inducing long term epigenetic changes which could affect the course of renal development and disease throughout the life course.

1.8 The Diet induced obesity mouse model

Animal models are invaluable for studying early life programming outcomes and mechanisms as discussed above, but one important limitation is that they are not always translatable to human disease. Now that maternal obesity is a common occurrence in the western world, it is important that animal models, as much as possible, accurately reflect the human condition if they are to be informative. To date, most animal models of maternal over-nutrition have used high fat feeding

(discussed in detail in chapter 3). Our laboratory has a C57BL/6 mouse model of maternal diet-induced obesity. This strain of mouse has a high susceptibility to obesity and hyperglycaemia (Breyer et al. 2009). We have generated multiple cohorts and have consistently showed that dams fed an *ad libitum* energy dense, palatable obesogenic diet, mimicking the common western diet in humans, become obese, hyperinsulinaemic and glucose intolerant (discussed in chapter 3).

For this PhD project, our model of maternal diet-induced obesity will be used to investigate the potential impacts on offspring renal health. Since this model closely mimics the characteristics of maternal obesity in western societies, it is hoped that any novel information gained pertaining to offspring renal health might contribute towards understanding the origins of kidney diseases in humans.

1.9 Hypothesis and Objectives

Hypothesis: Offspring exposed to maternal obesity during gestation and lactation will have altered renal morphology in early life and will go on to develop indicators of renal disease in later life. A post-weaning obesogenic diet will lead to renal damage in exposed offspring and will exacerbate renal damage in offspring exposed to maternal obesity.

In light of the above hypothesis, the overall aim of this thesis was to define the effects of maternal obesity on the offspring kidney and to determine which factors associated with maternal obesity might be responsible for kidney programming. This was achieved through the following objectives:

Objective 1 (chapter 3): To characterise the maternal physical and metabolic phenotype in a mouse model of maternal diet-induced obesity.

This was achieved by feeding dams an obesogenic diet and measuring body weight throughout pregnancy and lactation. Dam body composition was also assessed at mating and weaning. A glucose tolerance test was performed at weaning and organ weights as well as serum metabolites were assessed following weaning. These measures were compared to dams fed a control diet throughout the same period to define the maternal effects diet-induced obesity in pregnancy.

Objective 2 (chapter 4): To characterise renal morphology in male offspring exposed to maternal obesity at weaning (3 weeks of age).

This was achieved by assessing the cortex and medulla areas in kidneys of offspring exposed to maternal obesity. The total nephron number was determined using the physical dissector/fractionator combination. The glomeruli density (number of glomeruli/mm^2) was also measured. Finally, glomeruli

diameters were assessed as a proxy for glomeruli areas. These measures were compared to offspring exposed to a maternal control diet.

Objective 3 (chapter 5): To assess renal health and blood pressure in postpubescent male offspring (8 weeks of age).

This was addressed by using tail cuff plethysmography to measure blood pressure and heart rate in offspring exposed to maternal obesity. Renal morphological measures including medulla and cortex areas, glomeruli density and glomerular areas were also assessed. Urine and serum were analysed for indicators of renal function. Renal fibrosis was measured giving an indication of renal damage. These measures were compared to offspring exposed to a maternal control diet.

Objective 4 (chapter 6): To characterise the impact of maternal obesity on renal health with ageing and/or an offspring post-weaning obesogenic diet in adult male offspring (6 months of age).

This objective was achieved by assessing blood pressure and heart rate in offspring, as well as serum markers of renal function. To measure renal damage, the lipid content of offspring kidneys, as well as genes indicative of renal damage, inflammation and fibrosis were assessed. Finally, glomerulosclerosis and tubulointerstitial fibrosis were assessed by fibrosis staining. All measures were determined in offspring exposed to either a maternal obesogenic or control diet, and weaned onto either a control or obesogenic diet, in order to assess the effects of maternal obesity and post-weaning obesity on the offspring kidney independently.

Objective 5 (chapter 7): To determine whether maternal exercise in obese dams impacts on markers of renal development in 19 day old fetuses.

This was addressed by implementing a treadmill exercise intervention in obese dams during pregnancy. Gene and protein markers of renal development in fetuses exposed to maternal obesity with and without maternal exercise were then measured. These renal development markers were also correlated with maternal characteristics such as serum insulin to determine which maternal characteristics associated with obesity might be important in the programming of the offspring kidney.

Chapter 2. General Methods

All methods described in this chapter correspond to work that is presented in two or more chapters. Methods that are chapter specific are described in the individual chapter.

2.1 Generation of the animal model

All studies were approved by the University of Cambridge Animal Welfare and Ethical Review Board and were conducted according to the Home Office Animals (Scientific Procedures, UK) Act 1986. From weaning, females were either fed *ad libitum* standard laboratory chow (RM1) or a highly palatable obesogenic diet (45% AFE fat) (both diets from Special Dietary Services, Witham, UK). Females fed the obesogenic diet were also provided *ad libitum* sweetened condensed milk (Nestle, Croydon, UK) containing micronutrient mineral mix (AIN93G, Special Dietary Services, UK). Table 2.1 summarises the composition of both diets.

Table 2.1: *Composition of chow and obesogenic diets.*

Control Dams	**Obese Dams**	
RM1 pellets (10.74kJ/g)	**Obesogenic pellets (28.43kJ/g)**	**Condensed milk (13.7kJ/g)**
7% simple sugars	10% simple sugars	55% simple sugars
3% fat	20% animal lard	8% fat
15% protein	23% protein	8% protein
50% polysaccharide	28% polysaccharide	

At 6 weeks of age females were mated for a first pregnancy (P1). The presence of a copulatory plug confirmed females were pregnant. Females remained on their assigned diets throughout pregnancy and lactation. Females were housed one per cage and were kept in a controlled environment with 12 hour light and dark cycles and a constant temperature of 23^{o}C. At weaning, P1 litters were sacrificed by schedule 1 methods. This first pregnancy ensured females were proven breeders. Females were then kept on their respective diets for approximately 6 weeks until the body mass of “obesogenic” assigned females comprised at least 10g fat mass as measured by Time Domain- Nuclear Magnetic

Resonance (TD-NMR), (minispec TD-NMR, Bruker Optics, MA). Age matched control dams had less than 5g total fat mass.

Fresh food was administered every 2-3 days. Dams were weighed at day 0 (E0) and day 16 (E16) of pregnancy, and were subjected to TD-NMR at E0 and E16/E18. Pregnancy was complete after 21 days. 48 hours after birth (PN1), litters were culled to 6 pups with an equal sex ratio where possible. Dams were continued on their assigned diets throughout lactation. Both the dams and the litters were weighed on postnatal day 2 (PN2), PN7, PN14 and PN21 (3 weeks). At the end of lactation (PN21), male mice from both control and obesogenic dams were weaned onto either RM1 or the obesogenic diet creating 4 offspring groups. Only male offspring were studied so that results could be compared to previous findings from male offspring cohorts generated by this model in our laboratory. Additionally, males have been shown to be more susceptible to renal and cardiovascular diseases in both human studies and animal models (Patten 2007; Mahmoodzadeh et al. 2012; Blenck et al. 2016). Male mice were studied at 3 time points; 3 weeks, 8 weeks and 6 months. A schematic diagram of the model is presented in Figure 2.1.

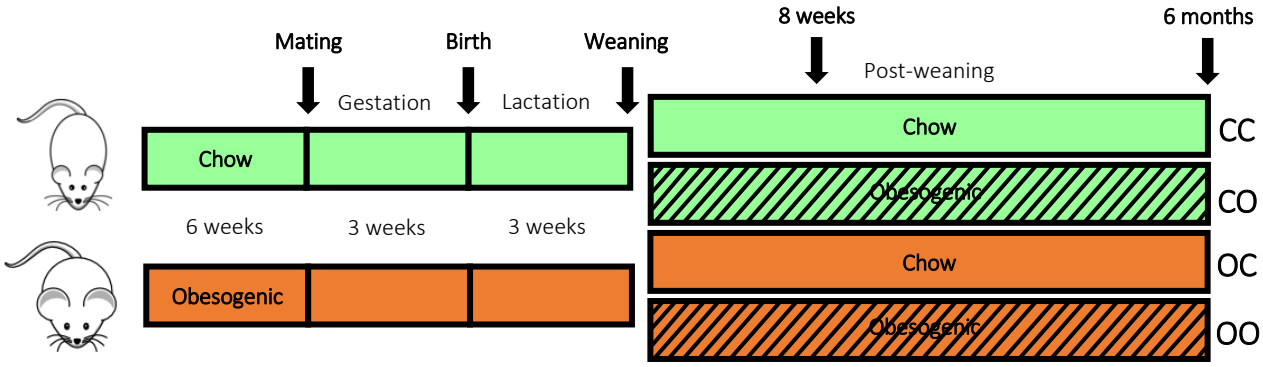


Figure 2.1. **A schematic detailing the study protocol.**

CC = offspring exposed to a *maternal* chow diet followed by a post-natal chow diet.
CO = offspring exposed to a *maternal* chow diet followed by a post-natal obesogenic diet.
OC = offspring exposed to a *maternal* obesogenic diet followed by a post-natal chow diet.
OO = offspring exposed to a *maternal* obesogenic diet followed by a post-natal obesogenic diet.

2.2 Blood glucose measurement

Glucose in blood collected from the tail vein was determined before culling dams and males at all time points using a glucose meter (Alpha Track, Illinois, USA).

2.3 Serum analysis

2.3.1 Serum collection

Blood was collected from dams following weaning and from 3 week, 8 week and 6 month old male mice. Mice were sacrificed by rising CO_2 asphyxiation and the blood collected by cardiac puncture. Blood was centrifuged for 2 x 4 min at 845 x g to separate serum (top phase) from the erythrocytes and clotting factors (bottom layer). Serum was then frozen at -80^{o}C for later analysis.

2.3.2 Serology

Non-haemolysed serum was sent to the mouse biochemistry phenotyping facility (Institute of Metabolic Science) for measurement of total cholesterol, High-density Lipoprotein (HDL), Low-density lipoprotein (LDL), triglycerides, insulin and leptin. Cholesterol and triglycerides were measured using an enzyme immunoassay with a Dade-Behring Dimension RXL analyser (Dade-Behring, Milton Keynes, UK). LDL cholesterol was calculated using the Friedwald equation, [LDL=Cholesterol - HDL - (Triglycerides/2.2)]. Insulin and leptin were measured by immunoassay using a Mesoscale Discovery MULTI-SPOT Assay System (Mesoscale Discovery, Maryland, USA). All samples were assayed in duplicate. Intra coefficients of variance used for quality control were: cholesterol 3.5%, triglycerides 4.5% and insulin 9%. Minimum detection levels were; cholesterol 1.3mmol/L, triglycerides 0.17 mmol/L and insulin 12.05pM/L.

2.4 Cardiovascular function

Systolic blood pressure and heart rate were measured in 8 week and 6 month old mice using a tail cuff plethysmography volume pressure recording system (BP-2000 Series II Blood Pressure Analysis System, Visitech systems). Conscious mice were placed in a restrainer on a heated pad and the tail was placed inside a tail cuff. Due to the dependence of blood pressure on the time of day, measurements were performed late in the afternoon on 3 consecutive days (between 4 and 5pm for

8 week mice and between 3 and 4pm for 6 month mice). The first 2 days were "familiarisation" days to allow the mice to acclimatise to the system and the data were not used for experimental analysis. The cuff was inflated and deflated a total of 15 times; the first 5 were practice measures and allowed the mice to adjust to the feeling of the cuff before the subsequent 10 cycles were recorded for later analysis.

2.5 Kidney histological analysis

2.5.1 Processing and Sectioning

Whole right kidneys from 8 week and 6 month old males were fixed in formalin immediately after dissection. After 48 hours, kidneys were transferred to 70% ethanol for storage prior to use. Kidneys were embedded in paraffin to preserve kidney morphology. The kidneys were then cut in half through the frontal plane. From the middle of each kidney, 4 sections were consecutively cut at 3µm and mounted onto positively charged slides. Sectioning is a specialised technique and was performed by a qualified histologist Mr Tom Ashmore.

2.5.2 Masson's trichrome staining

Masson's trichrome staining is a technique using 3 dyes used to differentiate between collagen and muscle fibers. Proteins and cells can be differentiated due to differences in structure and density of the protein networks. Smaller molecule dyes penetrate and stain erythrocytes, muscle and collagen. Medium molecule dyes stain only muscle and collagen, whereas larger molecule dyes can only penetrate and stain collagen, due to its open and porous structure (Suvarna et al. 2012). The Masson's trichrome staining technique is therefore ideal for assessing the extent of fibrosis within the kidneys.

Sections were deparaffinised in xylene (2x5 minutes) and rehydrated in 100% ethanol (2x2 minutes) before being re-fixed in Bouin's solution overnight at room temperature in order to intensify masson's staining. Sections were then washed before staining with Weigert's iron haematoxylin solution for 10 minutes to stain nuclei violet. The sections were washed again before Biebrich scarlet-acid fuchsin solution was added for 10-15 minutes, staining all acidophilic matrix including collagen, and cytoplasm red. Sections were washed in distilled water before differentiation in phosphomolybdic-phosphotungstic acid solution for 10-15 minutes to remove the red colour from collagen. When collagen fibres were no longer red, sections were transferred to aniline blue solution for 5-10 minutes to re-stain collagen blue. Sections were rinsed in distilled water before differentiating in 1% acetic acid

for 2-5 minutes to intensify the different stains. Finally sections were washed in distilled water and rapidly dehydrated through ethanol changes before being cleared in xylene and mounted using pertex mounting medium (CellPath, Newtown, UK) and glass cover slips (VWR, Lutterwoth, UK).

2.5.3 Periodic Acid Schiff staining

Periodic acid-Schiff (PAS) staining is a widely used technique for the demonstration of carbohydrates or glycoconjugates. In particular, the reactivity of Schiff reagent with glycoproteins of the basal lamina makes it a valuable technique for assessing basement membrane expansion within glomerular capillaries and tubules of the kidney (Suvarna et al. 2012).

Sections were deparaffinised in xylene (2x5 minutes) and rehydrated in 100% ethanol (2x2 minutes) before being submersed in 1% periodic acid (Sigma Aldrich, Gillingham, UK) for 10 minutes to oxidise tissue carbohydrates. The sections were rinsed in several changes of distilled water before being immersed in Schiffs reagent (Sigma Aldrich, Gillingham, UK) for 20 minutes in order to stain glycoproteins magenta. Tap water was used to rinse the sections for 10 minutes before adding haematoxylin for 30 seconds to stain the nuclei violet. The sections were rinsed with tap water before dehydrating in ethanol changes and mounting using resinous mounting medium and glass cover slips.

2.5.4 Imaging

PAS and Masson's stained sections were imaged at 20-fold magnification using a Zeiss Axioscan Z1. Zen 2.3 lite was used to visualise and prepare images for fibrosis analysis. Sample identification was blinded prior to analysis. Pictures of glomeruli were taken at random by zooming into the original images by 80%. Pictures of the cortex were taken by zooming into the original images by 40%. Random, non-overlapping images were captured from each sample.

2.5.5 Fibrosis analysis

2.5.5.1 Image conversion

Figure 2.2 describes how Fiji (ImageJ) was used to set a stain intensity threshold for images of PAS and masson's stained cortex and glomeruli. Intensities between this threshold were measured as fibrosis. Sclerosis was expressed as the % fibrotic tissue.

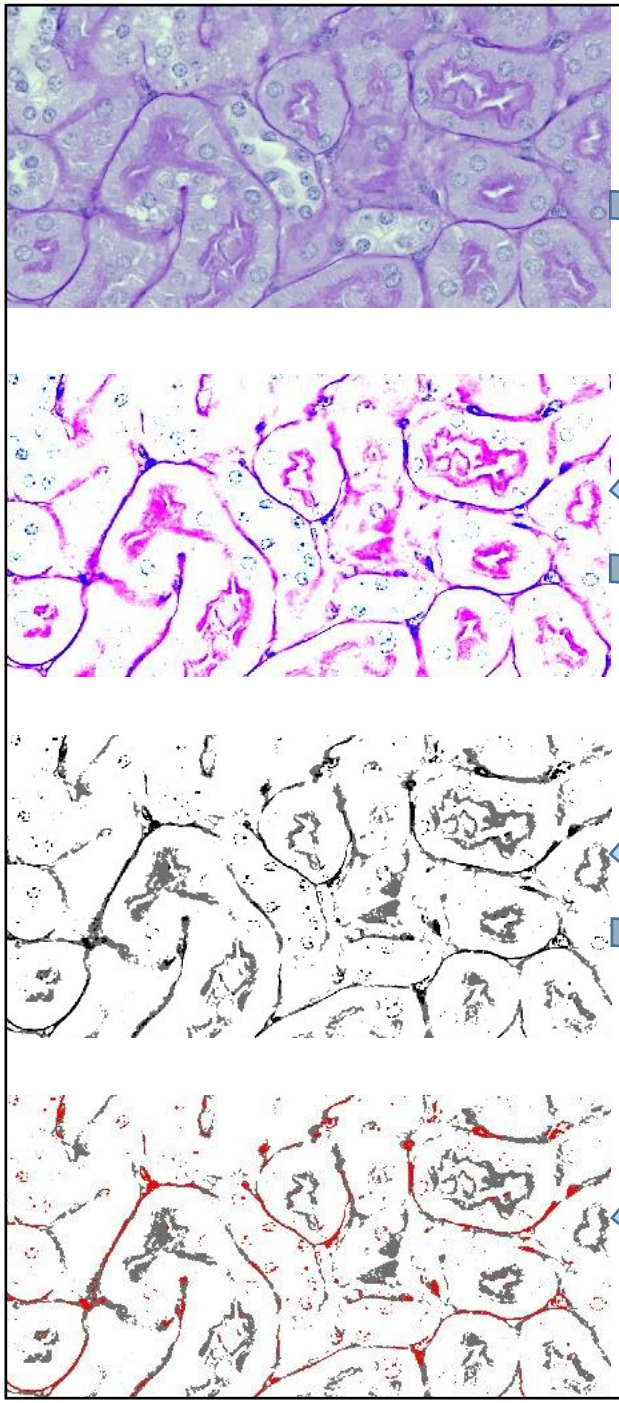
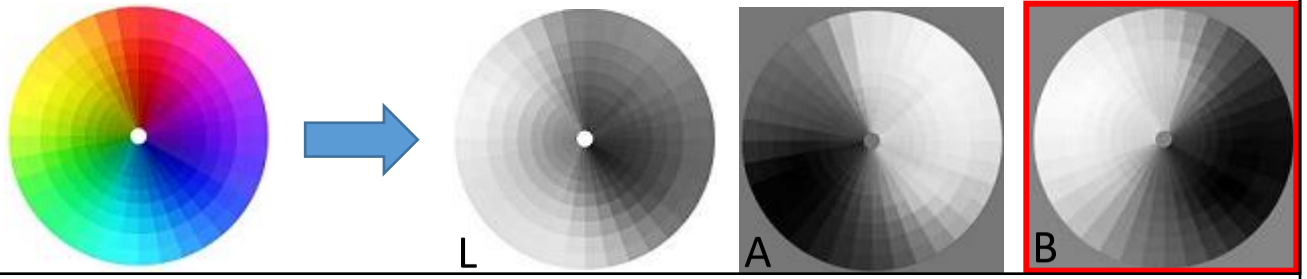


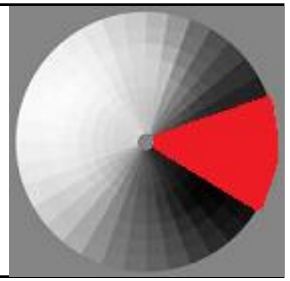
Figure2.2. **Image conversion and fibrosis quantification using ImageJ.** The images of the cortex on the left show an example of how images were changed throughout the process. The text and corresponding images explain step by step how images were changed and how fibrosis was quantified.

The minimum and maximum intensities in the image were found. A proportion of the maximum intensity was used to set a new signal range. Signals that were outside of this range were removed.

Numerical values were assigned to the signal intensities in the image.
The image was converted to greyscale (L), red to green scale (A) and yellow to blue scale (B). The yellow to blue scale was selected, transforming pinks and blues in the image to greys and black.



Using a proportion of the new minimum and maximum intensities, a threshold was defined. Signals with an intensity between this threshold can be seen in red. Red areas were deemed fibrotic and quantified as a percentage of the total image (cortex) area.



2.5.5.2 Quantifying glomerulosclerosis

After image conversion, the freehand selection tool was used to define the glomerular tuft area as shown in Figure 2.3. The % fibrosis (red) coverage was measured within the selected area. Over 20 PAS and Masson's stained glomeruli were measured per sample. A Grubb's test was performed using QuickCalcs (GraphPad) in order to exclude outliers before the mean % fibrosis was calculated for each sample.

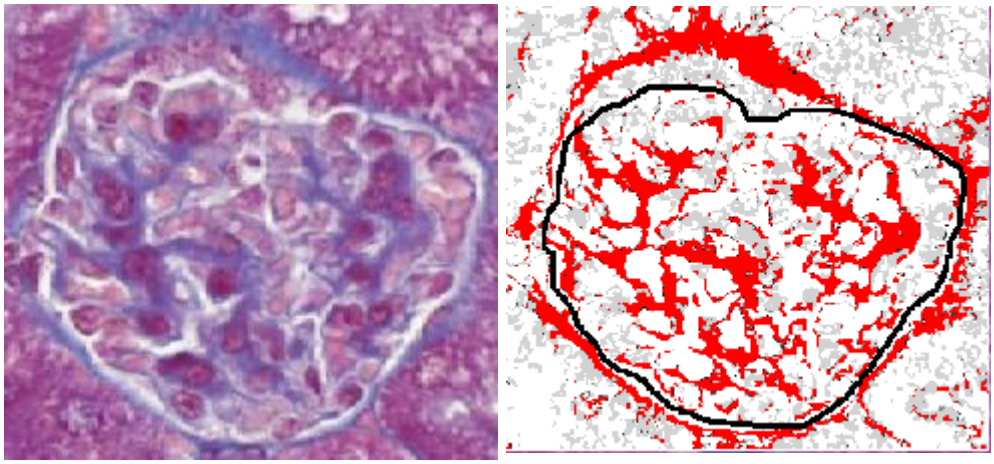


Figure 2.3. **An example of glomerulosclerosis quantification.** The left hand picture shows a Masson's stained glomerulus before image conversion. The right hand image shows the same glomerulus after image conversion. The black line was drawn using the Bowman's space as a guide. The area within the black line is the glomerular tuft and represents the area that % fibrosis was quantified.

2.5.5.3 Quantifying tubulointerstitial fibrosis

Using the polygon selection tool on ImageJ, glomeruli were excluded from images of the cortex before measuring the % fibrosis. Over 20 PAS and masson's stained images were measured per sample. Outliers were excluded before the mean % fibrosis was calculated for each sample.

2.5.6 Glomeruli area quantification

PAS stained images taken on the Axioscan were analysed using image analysis software (Halo, Indica Labs, Corrales, USA). Starting from where the cortex meets the area including the renal pelvis, artery, vein and ureter, and working counter- clockwise, every glomerulus area was measured for each sample, until over 100 glomeruli areas were determined (Figure 2.4). Glomeruli areas were measured

using the pen annotation mode; the glomeruli tufts were precisely drawn around giving the total area within (Figure 2.5).

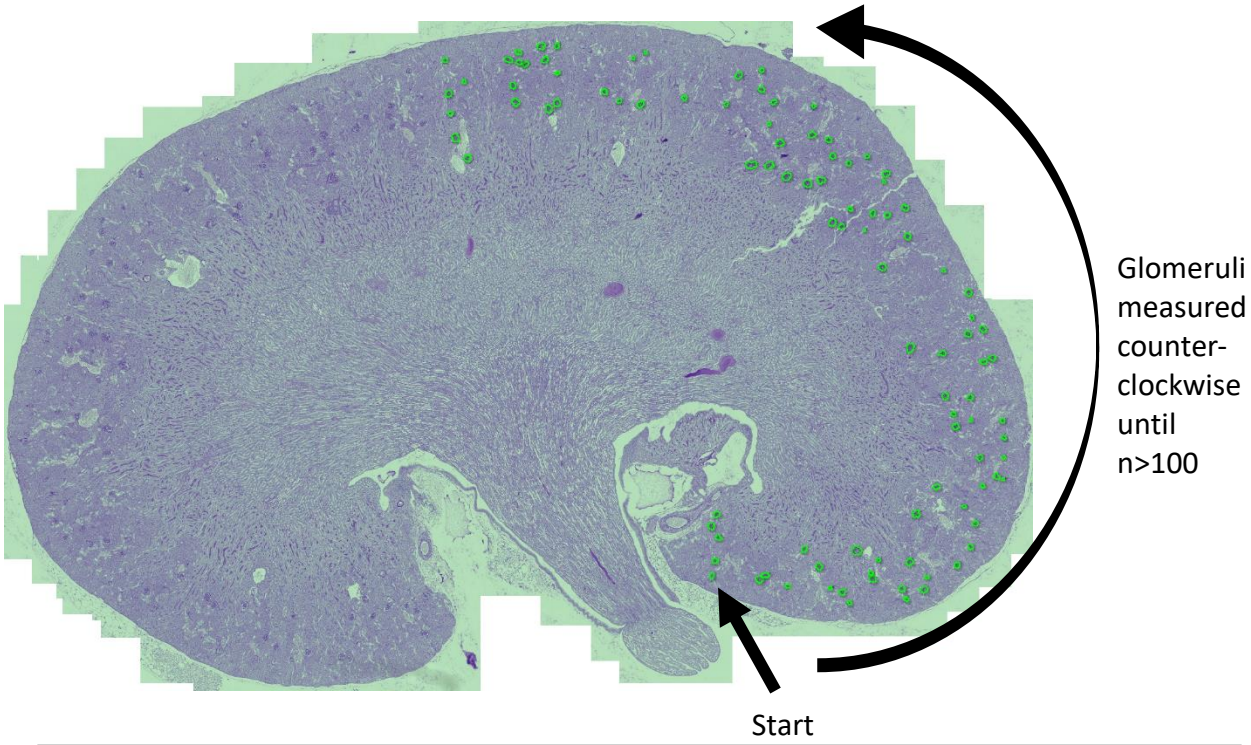


Figure 2.4. **Glomeruli area quantification.** The pen annotation mode (Halo, Indica Labs) was used to draw around every glomerulus starting from the renal pelvis and working counter-clockwise.

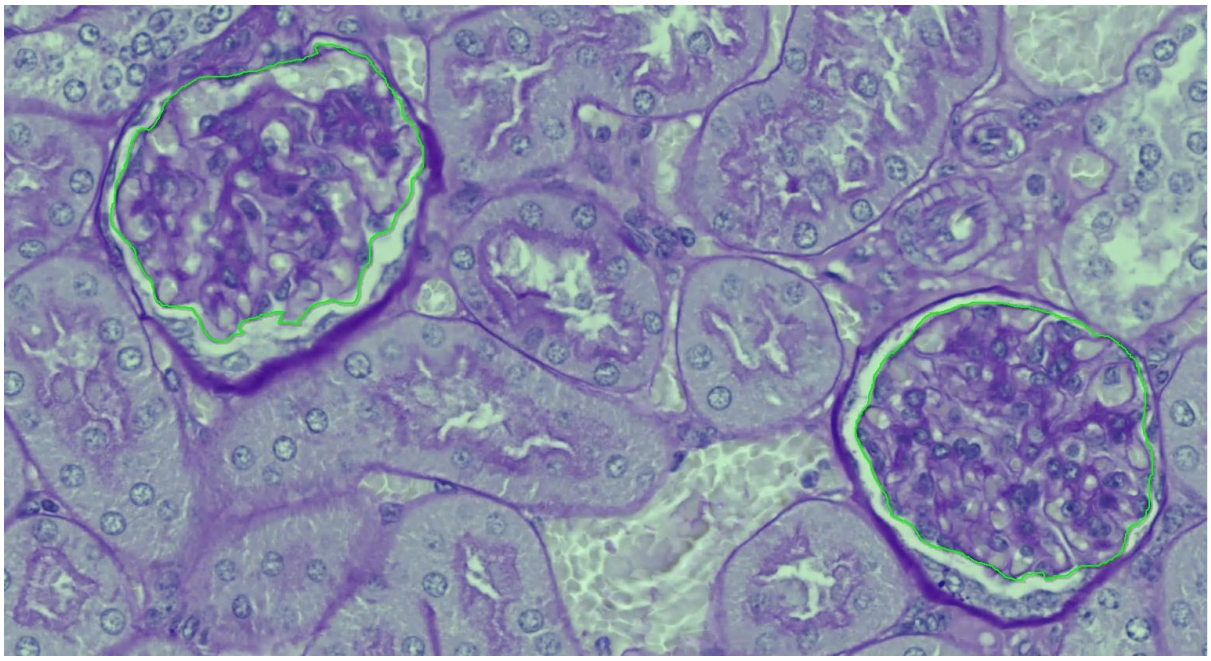


Figure 2.5. **Glomerulus area quantification method.** The pen annotation mode (Halo, Indica Labs) was used to draw around each glomerular tuft giving the area (μm^2) within.

2.5.7 Medulla and cortex area and glomeruli density measurement

PAS stained images taken on the Axioscan were analysed using image analysis software (Halo, Indica Labs, Corrales, USA). Using the pen annotation mode, the cortex and medulla on each section was precisely drawn around, giving the total area within. The pen annotation mode was also used to mark every glomerulus on each section. Glomeruli density was then calculated as total number of glomeruli divided by the total area.

2.6 Quantifying mRNA expression

2.6.1 Tissue Rupture

Whole mouse kidneys from E19 offspring and 6 month old males were stored at -80^{o}C. Kidneys were powdered on dry ice using a pestle and mortar. 15mg tissue from each kidney was placed into a RNA free eppendorff. RNA was extracted using a miRNeasy Mini Kit (QIAGEN, Hilden, Germany). 700µl QIAzol lysis reagent was added to each sample (roughly 47 times the volume of tissue mass) and homogenised using a motorised tissue rupture (Qiagen, Sussex, UK).

2.6.2 Organic extraction

The resulting homogenate was incubated at room temperature for 5 minutes before adding 140µl chloroform. Each sample was shaken vigorously for 15 seconds to mix before incubating at room temperature for 2-3 minutes. The homogenate was centrifuged for 15 minutes at 12000 x g at 4^{o}C. Centrifugation separated the organic and aqueous phases of the lysate. The aqueous phase (300µl) was placed into a new RNA free tube.

2.6.3 RNA Isolation

1.75x the volume (525µl) of 100% ethanol was added to each sample and mixed thoroughly. 700µl of the ethanol/lysate solution was then added to an RNeasy Mini spin column within a 2ml collecting tube and centrifuged for 15 seconds at 8000 x g at room temperature. The flow through was discarded. 30ml ethanol was added to 15ml RWT buffer concentrate and 44ml ethanol added to 11ml RPE buffer concentrate before use. 700µl RWT wash buffer was added to the mini column and centrifuged for 15 seconds at 8000 x g. The flow through was discarded. 500µl RPE wash buffer was then added to each mini column and centrifuged for 15 seconds at 8000 x g. The flow through was discarded. 500µl RPE

wash buffer was added to each mini column and centrifuged for 2 minutes at 8000 x g, the flow through was discarded. Each mini column was transferred to a new 2ml collection tube before centrifuging at maximum speed (20238 x g) for 1 minute to completely dry the filter within the column. The mini columns were transferred to new 1.5ml collection tubes and 30ul RNA free water was pipetted into each filter before centrifuging for 1 minute at 8000 x g to elute RNA. The resulting RNA eluent was placed on ice until quantification.

2.6.4 RNA quantification

The concentration of RNA from each sample was quantified using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Waltham, USA). Using the RNA nucleic acid setting**,** 1.5µl nuclease free water was used to set a baseline null measurement. 1.5µl RNA was then added to quantify the concentration of RNA in each sample (ng/µl). If the sample contained pure RNA a single peak would be observed at a wavelength of 260nm with a concentration greater than 100ng/µl. The 260/280 ratio was used to assess RNA purity, a value of 2.0 indicated pure RNA. A value lower than 2.0 indicated potential contamination of protein or other contaminants. All samples had a 260/280 ratio of above 2.0. The presence of ethanol and phenol contaminants was assessed by the 260/230 ratio, a value between 1.8- 2.2 indicated clean RNA. All samples included in the analysis fulfilled this criteria. RNA was then diluted to a concentration of 100ng/µl with DNase free H_2O. The new concentration was confirmed using the NanoDrop ND-1000 Spectrophotometer.

2.6.5 RNA integrity

RNA integrity was assessed by denaturing gel electrophoresis. 1g agarose (1% gel) was heated in 72ml water until dissolved. Once cooled below 60^0C, 10 ml 10x 3-(N-morpholino) propanesulfonic acid (MOPS) (0.4M MOPS, 0.1M sodium acetate, 0.01M Ethylenediaminetetraacetic acid (EDTA) (pH 7.0)) running buffer and 18ml 37% formaldehyde was added. 10µl of SYBRsafe (Lifetechnologies, Paisley, UK) DNA gel stain was added to the agarose (Bioline, London, UK) and MOPS/formaldehyde buffer mixture. The buffer was poured into the gel tank with a comb in place. Once the gel had set, 1xMOPS buffer was poured into the tank, covering the gel. The comb was removed and RNA HyperLadder 1kb (Bioline, London, UK) was loaded into the first well. RNA samples were prepared by mixing 3µl RNA (concentration 100ng/µl) with 5µl loading buffer and 2µl DNAase free water. All RNA samples were loaded and the gel was run at 80 volts for 40-80 minutes. After running, the gel was visualised using an imager (BioRad, ChemiDoc XRS+) and a picture was take (Figure 2.6 shows representative gel).

Samples that failed to show clear 28S and 18S rRNA bands and/or had signs of smear were excluded from quantitative Real-Time Polymerase Chain Reaction (qPCR) analysis.

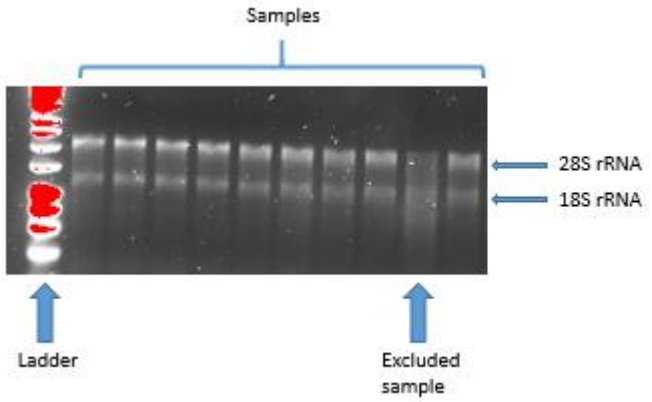


Figure 2.6. **Image of an RNA gel.** Samples with clear 18S and 28S rRNA bands were included in qPCR analysis.

2.6.6 cDNA synthesis

Reverse transcription was performed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Paisley, UK) (Table 2.2).

Table 2.2. *Reagents and their respective volumes required for an RNA to cDNA reaction.*

Reagents provided in kit	**µl for 1 reaction**
10x RT buffer	2
25x dNTP mix	0.8
10x random primers	2
Multiscribe RT	1
RNase inhibitor	1
Nuclease free H_20	9.2

16µl of the master reaction (Table 2.2) was pipetted into RNase free tubes. 4µl RNA (100ng/µl) was added and mixed by pipetting. The resulting master reaction/RNA samples were centrifuged for 10 seconds at 10,000 x g to collect samples at the bottom of the tubes and then placed in a thermal cycler (Applied Biosystems Veriti 96 well, Paisley, UK) to perform the reverse transcription reaction (Table 2.3). 10µl of the resulting 20µl cDNA was diluted with 190µl water (1 in 20 dilution). Concentrated and diluted cDNA was stored at -20°C.

Table 2.3. *The steps required for reverse transcription.*

	Step 1	**Step 2**	**Step 3**	**Step 4**
Process	Denaturation	Annealing	Elongation	Inactivation
Temperature (°C)	25	37	85	4
Time (minutes)	10	120	5	

2.6.7 Primer design

Mus musculus genes were located on the NCBI-Gene website (URL: http://www.ncbi.nlm.nih.gov/gene). The NM_ number for each gene was inputted into primer blast (URL: http://www.ncbi.nlm.nih.gov/tools/primer-blast/). A product size of 70-200 nucleotides was selected. Primers were also required to span exon-exon junctions to prevent amplification of genomic DNA. These selection criteria then resulted in a number of primers on the intended gene target. Primers with unintended targets were excluded. Primers with the lowest potential for 3' self-complementarity, as assessed on https://www.sigmaaldrich.com/pc/ui/tube-manual-entry?product=standard, were then selected. Information regarding selected primers can be found within the relevant chapters.

2.6.8 Quantitative Real Time Polymerase Chain Reaction (qPCR)

mRNA expression was quantified using Sybr Green and either the StepOne plus qPCR engine (Life Technologies, Bromborough, UK) for E19 samples (96 well plates) or the Applied Biosystems 7900HT Fast Real-Time PCR System for 6 month samples (384 well plates). The steps involved in the real time qPCR reaction for both machines in presented in Table 2.4.

Table 2.4. *The steps required for qPCR.*

	Holding stage	**Cycling stage (x40)**		**Dissociation stage**		
Temperature (°C)	95	95	60	95	60	95
Time (minutes)	10	0.15	1	0.15	1	0.15

2.6.9 Primer validation

A primer mix (0.5µmol) for each gene was made by adding 2.5µl each of forward and reverse primers (100µmol) to 495µl DNase free H_20. Primer efficiency was validated using a cDNA standard (see below). 10µl diluted (1 in 20) cDNA from each sample was pooled to create the standard (S1) that was then serially diluted to create 6 standards. 2.5µl cDNA standard and 9.5µl sybr green/primer (1µl primer mix, 6µl sybr green and 2.5µl H_20) was added to each well within either a 96 or 384 well plate. cDNA standards were loaded in duplicate. Primers were validated by;

1. Cycle threshold (CT) values for the pooled standard (S1) between 20-31.
2. Presence of a single peak on the melt curve at the correct melting temperature (Figure 2.7).
3. Efficiency of the reaction between 90-110% to ensure doubling of the CT value with every reaction cycle.

Primers which failed to meet the above criteria were not used for mRNA expression analyses.

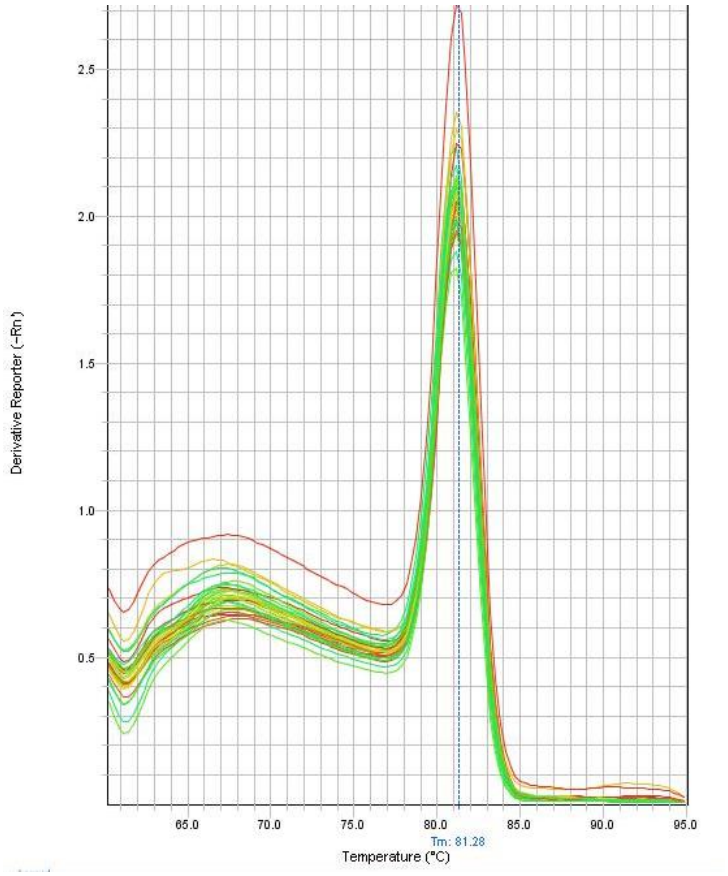


Figure 2.7. **Example of melt curve.** The presence of a single peak indicated that the specific target product was amplified.

2.6.10 Analysing mRNA expression

The expression of all genes of interest were calculated using the ΔΔCt method by normalising the Ct of the gene of interest to the combined geomean of *Hprt*, *Gapdh* and *Sdha*. The housekeeping genes were validated as ideal "housekeepers" as maternal and offspring diets had no effect on expression as assessed by 2 way ANOVA (for 6 month samples) or 1 way ANOVA (for E19 samples) ($p>0.05$).

2.7 Statistics

All statistical analyses were performed using Prism 5 (Graphpad). All data were checked for normality before making statistical comparisons. For data with 2 groups, normally distributed data were analysed by students T test. Non-normal data were analysed by Mann-Whitney U test. Data comprising 4 groups were analysed using 2-way analysis of variance (ANOVA).

Chapter 3. The maternal diet-induced obesity mouse model.

3.1 Introduction

3.1.1 Why study maternal obesity?

Obesity has reached epidemic levels globally in recent years and as such, it is now a common complication affecting women of reproductive age, particularly in western societies. In the US and the UK, 50% of women are overweight or obese during pregnancy (Richter et al. 2015), and 20 -40% gain more weight during the course of pregnancy than is recommended (Thangaratinam et al. 2012). In the short term, maternal obesity increases the risk for adverse pregnancy complications including hypertension, pre-eclampsia and caesarean delivery (Ching et al. 2016). Importantly, there is now substantial evidence from epidemiological studies that maternal obesity predisposes exposed individuals to adverse health consequences. Maternal BMI or GWG has been shown to correlate with offspring adiposity and blood pressure (Lawlor et al. 2004), poor metabolic profile (Gaillard et al. 2015) and T2DM (Dabelea et al. 2008) in both children and adolescents. These early health complications lead to poor outcomes in later life. It was found that hospital admissions due to cardiovascular events, as well as all-cause mortality rates, were increased in 34-61 year old individuals who had been exposed to maternal obesity (Reynolds et al. 2013). Eriksson and colleagues also found, in a cohort of Finnish men, that the risk of death from CHD correlated with maternal BMI (Eriksson et al. 1997).

Maternal obesity is also an important risk factor for GDM. Women who are obese before pregnancy are four times more likely to develop GDM than women of normal weight (Chu et al. 2007). Individuals exposed to GDM show similar phenotypes to those exposed to maternal obesity. Increased central adiposity (Lawlor et al. 2010), increased BMI and blood pressure (Bunt et al. 2005; Tsadok et al. 2011), and T2DM (Franks et al. 2006; Clausen et al. 2008; Dabelea et al. 2008) have all been documented to be increased in individuals exposed to maternal GDM.

It could be argued that abnormal metabolic phenotypes can be passed from mother to child through shared genetics. However, with respect to maternal obesity, it has been observed that children born to mothers following weight loss due to bariatric surgery show a 50% decrease in obesity compared to their siblings born pre maternal weight reducing surgery (Kral et al. 2006). Likewise, with respect to maternal GDM, individuals born after the onset of maternal diabetes are significantly more likely to develop T2DM when compared with siblings born before the onset of maternal GDM (Dabelea et al. 2000). These observations solidify the role of the early life metabolic environment *per se* on the health outcomes of future generations, independent of genetic factors.

The outcomes observed in individuals exposed to maternal obesity and GDM highlight the need to investigate how impaired metabolic health is passed from one generation to the next. Ultimately, this may aid in the development of interventions that can prevent obesity in future generations, and end a vicious cycle which has led to the exponential growth in obesity prevalence in recent years.

3.1.2 Why generate a mouse model of diet-induced obesity?

Animal models provide a valuable tool for researching the mechanisms associated with the early life programming of adverse health, since they allow for the manipulation and tight control of environmental and genetic factors. In general, animal models of maternal over-nutrition lead to outcomes in offspring which are similar to those seen in epidemiological studies. This is discussed in more detail in chapter 1, section 1.5.3. However, the ability to uncouple maternal characteristics in animal models, (which would usually be interconnected in humans) to remove confounding factors has also highlighted that subtle environmental changes can have important consequences for offspring health and helped in the identification of "programming factors" associated with maternal obesity that could mediate detrimental effects in the offspring. With respect to animal models of maternal over-nutrition, slight changes in dietary composition, the timing of exposure, as well as associated maternal characteristics such as impaired glucose tolerance and insulin resistance have the potential to affect offspring outcomes [reviewed in (L. Williams et al. 2014)]. For example, in a non-human primate model of maternal high fat diet, offspring showed a three-fold increase in liver triglycerides and evidence of the development of non-alcoholic fatty liver disease (McCurdy et al. 2009). However, this phenotype could be mainly reversed by a change in maternal diet which did not result in a change in maternal weight (McCurdy et al. 2009). Similarly, Frihauf and colleagues used a model that utilized genetic strains of mice that were either resistant or susceptible to diet induced obesity. Females were then fed either a control or western diet throughout pregnancy and lactation. Offspring exposed to a maternal western diet showed increases in adiposity despite mothers showing a normal energy intake, body weight and pregnancy weight gain (Frihauf et al. 2016). Conversely, obesity in mouse mothers, induced by over-eating a control diet, also appeared to induce metabolic perturbations in the offspring. Importantly, the combination of maternal obesity and a western diet produced an additive effect on offspring insulin and leptin levels. These observations emphasise that both obesity and dietary composition are important and independent programing factors that influence offspring outcomes.

The findings above emphasise the need for an animal model which typifies maternal obesity in western societies, in order to attain valuable, translatable results. Our laboratory has established a

mouse model of maternal obesity induced by *ad libitum* feeding of an energy dense (high in sugar and fat) palatable diet pre-pregnancy and during pregnancy and lactation. Our laboratory has consistently demonstrated that dams exposed to the obesogenic diet are heavier than control mice throughout pregnancy and lactation and that this is due to an increase in fat mass, with dams having approximately double the level of adiposity of controls (Fernandez-twinn et al. 2012; Blackmore et al. 2015; Fernandez-Twinn et al. 2017). Obese dams also show metabolic abnormalities including glucose intolerance, raised insulin and leptin levels (Samuelsson et al. 2008; Fernandez-Twinn et al. 2017). Our model is highly reproducible, and can be considered to reflect human maternal obesity in the western world where diets are rich in fats and sugars and often associated with metabolic dysfunction such as GDM.

The data presented in this chapter describes the phenotype of dams throughout pregnancy and lactation, used for offspring studies during this PhD. It is important to understand the maternal phenotype in order to define which component of maternal obesity can programme offspring kidney dysfunction and to help in the design of rational intervention strategies.

3.2 Aims

The aims of this chapter were:

1. To characterise the phenotype of control and obese dams throughout gestation and lactation.
2. To assess glucose tolerance, insulin and lipid levels in control and obese dams at the end of lactation.

3.3 Methods

A thorough description of the generation of the animal model can be found in chapter 2, section 2.1.

3.3.1 Dam phenotyping

Dams were weighed weekly from gestational day 1 (E1) until E14. Dams were then left undisturbed in order to litter, and weighed weekly from post-natal day 7 (PN7) until the end of lactation. Body composition was determined before mating and again at weaning by TD-NMR. Solid food (high fat diet and chow) was weighed weekly, and milk was weighed and changed twice weekly, during gestation and lactation in order to calculate dam food intake. Measures of metabolic function were not conducted during pregnancy since this may have been stressful for dams, introducing confounding factors which could have affected later offspring studies. Dam serum analyses were therefore conducted at the end of lactation. After pups were weaned, dams underwent a 4 hour fast before an intraperitoneal glucose tolerance test (IPGTT) was performed. Briefly, glucose (1g/kg dam weight) was injected into the peritoneum. Glucose levels were then measured in blood taken from the tail vein using a glucose meter (Alpha Track, Illinois, USA). Glucose levels were determined at 0, 15, 30, 45, 60, 90 and 120 minutes following the initial glucose injection. IPGTT is best performed with two people so was conducted in collaboration with Dr Laura Kusinski. Following weaning, dams remained fed (for organ weights and serum insulin measurement), or were fasted overnight (for serum lipid analysis) and then killed by rising CO_2 asphyxiation. Blood was collected by cardiac puncture and prepared and stored as described in chapter 2, section 2.3.1. Organs and fat pads were dissected, weighed and stored at -80^{o}C. Serum lipid levels were determined as described in Chapter 2, section 2.3.2. Insulin levels were then determined by enzyme linked immunosorbent assay (ELISA) (Mercodia, Uppsala, Sweden). The lower level of detection for the kit was 22.62pmol/l.

3.3.2 Offspring phenotyping

Two days after birth, pups were sexed, the number of pups in each litter was recorded and then standardised to six pups. The whole litter weight was determined on PN3. The average offspring weight was then calculated.

3.3.3 Statistics

All statistical analyses were conducted using Prism 5.0 (GraphPad, CA, USA). All data were checked for normality by either a Shapiro-Wilk or a Kolmogorov–Smirnov (for smaller data sets) test. Normally distributed data were analysed by students T tests and the mean and the standard error of the mean (SEM) are presented. Non-normal data (serum triglycerides and the number of pups) were analysed by Mann-Whitney U test and the median and interquartile range (IQR) are presented. For all data sets, $p<0.05$ was considered statistically significant. Sample sizes (n) are reported within each figure where n indicates the number of dams represented.

3.4 Results

Dams fed the obesogenic diet were significantly heavier at mating and throughout gestation and lactation, when compared with control dams (Figure 3.1.A). The difference in body weight between the groups was consistent throughout this period, indicating that weight gain during pregnancy and weight loss after pregnancy was similar between groups. The increase in weight of dams fed the obesogenic diet was due to an increase in fat mass and not lean mass. Obesogenic diet fed dams showed a significant increase in fat mass at mating (Figure 3.1.B), and at weaning (Figure 3.1.C). Whereas lean mass was slightly but significantly decreased in dams fed the obesogenic diet at mating (Figure 3.1.B), and at weaning (Figure 3.1.C).

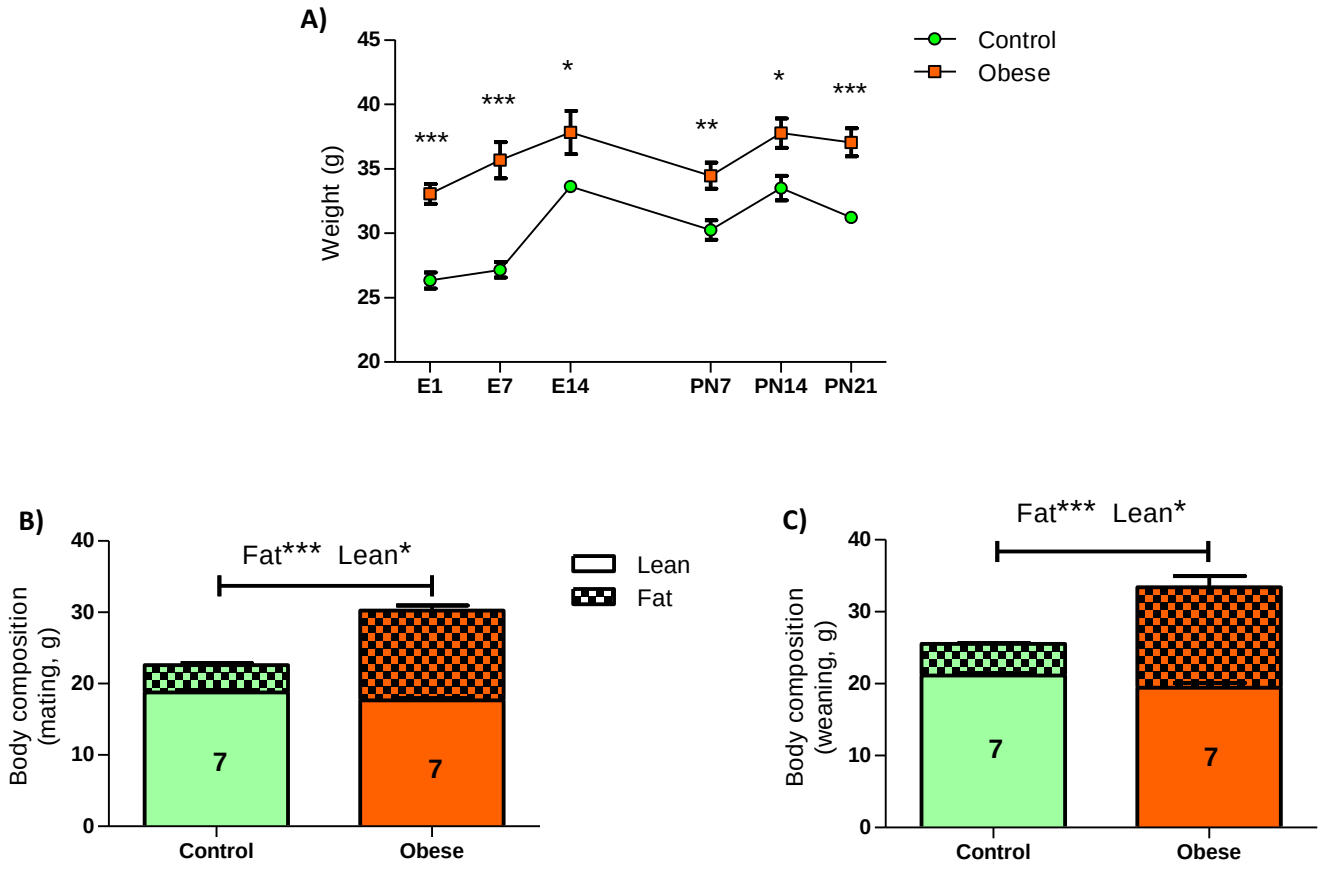


Figure 3.1. **Maternal weight and body composition. A)** Maternal weight throughout pregnancy and lactation. N=7 control, 7 obese. **B)** Maternal body composition at mating. **C)** Maternal body composition at weaning. N numbers are presented within the graphs. Students T test; *p<0.05, ***p<0.0001.

Following weaning, there was no difference in absolute heart weight or heart weight normalised to lean mass between control and obese dams. When normalised to body weight, the heart weight of obese dams was significantly decreased when compared with control dams. There was no difference in absolute total kidney weight between obese and control dams. Kidney weight relative to body weight was significantly decreased in obese dams, whereas kidney weight normalised to lean mass was increased in obese dams. Liver weight (absolute and normalised to body weight) was unaffected by maternal diet, however when normalised to lean mass, obese dams had an increased liver weight. There were no significant differences in ovary weight or uterus weight (absolute, relative to body weight and lean mass) between obese and control dams. Obese dams showed an increase in absolute retroperitoneal fat, intra-abdominal fat and ovarian fat. These increases were maintained in obese dams when fat pads were normalised to body weight and lean mass. These results are summarised in Table 3.1.

Table 3.1. *Maternal fed post-weaning organ weights.*

Absolute organ weights (mg)	**Control (n=7)**	**Obese (n=7)**
Heart	169±6	168±6
Total kidney	385±9	396±9
Liver	2062±64	2362±158
Total ovary	10±1.4	12.1±0.9
Total uterus	78.8±7.3	91.7±10.5
Retroperitoneal fat	100±6	497±47***
Intra-abdominal fat	856±108	3102±303***
Ovarian fat	90.6±10.8	530.6±79.9***
Organ weights (% of body weight)	**Control (n=6)**	**Obese (n=7)**
Heart	0.57±0.02	0.42±0.02***
Total kidney	1.25±0.03	0.99±0.04***
Liver	6.89±0.27	5.7±0.45
Total ovary	0.034±0.005	0.029±0.003
Total uterus	0.25±0.03	0.22±0.03
Retroperitoneal fat	0.33±0.02	1.28±0.04***
Intra-abdominal fat	2.96±0.4	7.3±0.17**
Ovarian fat	0.28±0.03	1.25±0.16***
Organ weights (% of lean mass)	**Control (n=7)**	**Obese (n=7)**
Heart	0.8±0.03	0.89±0.05
Total kidney	1.78±0.07	2.0±0.04*
Liver	9.76±0.31	11.85±0.73*
Total ovary	0.047±0.006	0.061±0.005
Total uterus	0.37±0.04	0.46±0.06
Retroperitoneal fat	0.48±0.03	2.52±0.24***
Intra-abdominal fat	4.06±0.5	15.54±1.41***
Ovarian fat	0.43±0.06	2.66±0.4***

N numbers are presented within the table. Students T test; **p<0.001, ***p<0.0001.

During gestation and lactation, dams fed the obesogenic diet had an increased food intake relative to dams on the control diet. The amount of food consumed, measured in grams per day, was increased in dams on the obesogenic diet (Figure 3.2.A). This led to 3-fold increase in the daily kilocalories consumed by obese dams when compared to control dams (Figure 3.2.B).

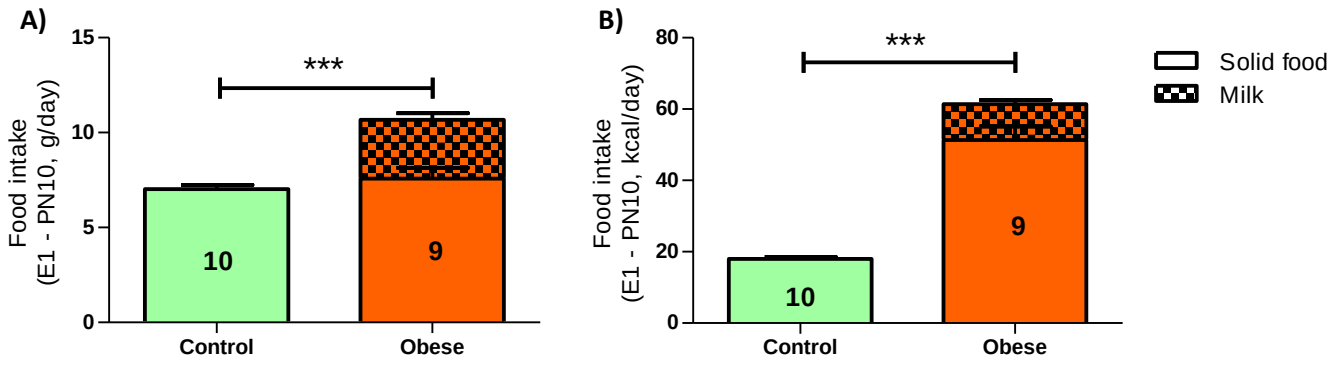


Figure 3.2. **Maternal food intake during gestation and lactation**. **A)** Weight of food consumed by dams per day. **B)** Kilocalories consumed by dams per day. N numbers are presented within the graphs. Students T test; ****p*<0.0001.

At weaning, control and obese dams showed no differences in their glucose tolerance in response to an IPGTT (Figure 3.3.A). The IPGTT area under the curve was similar between groups (Figure 3.3.B). However, following weaning obese dams showed a significant increase in serum insulin levels relative to control dams (Figure 3.3.C).

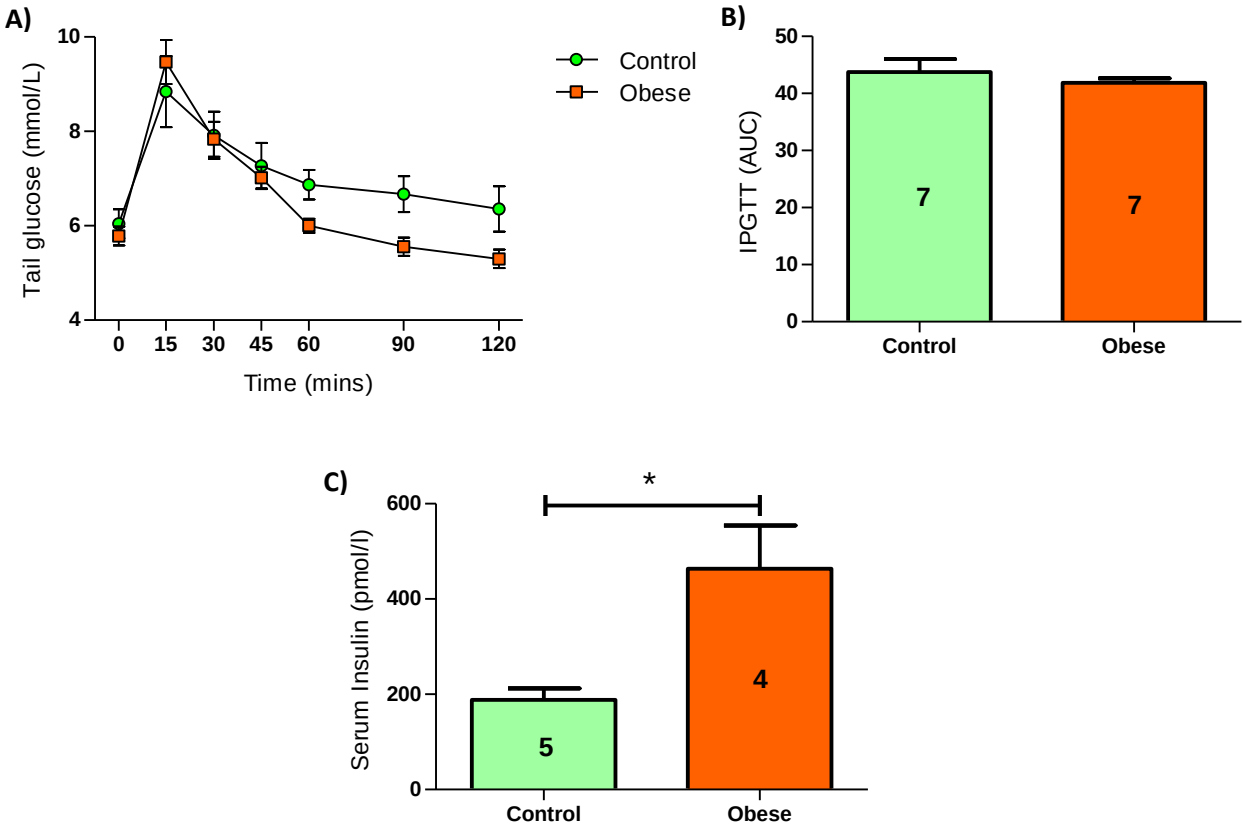


Figure 3.3. **Maternal glucose and insulin levels at weaning**. **A)** Maternal tail glucose levels during an IPGTT. N= 7 control, 7 obese. **B)** The IPGTT area under the curve. **C)** Maternal serum fed insulin levels. N numbers are presented within the graphs. Students T test; *p<0.05.

Following weaning, there was no difference in serum triglycerides between control and obese dams (*p*=0.95). Obese dams showed a significant increase in free fatty acid (FFA) and total cholesterol levels compared to control dams. These results are summarised in Table 3.2.

Table 3.2. *Maternal fasting serum lipids post-weaning*.

Serum lipid	**Control (n=7)**	**Obese (n=7)**
Triglycerides (mmol/l)	0.8±0.4	0.7±0.6
FFA (µmol/l)	411±45.6	796±130*****
Total cholesterol (mmol/l)	2.11±0.14	3.76±0.19*******

N numbers are presented within the table. Mann Whitney test (Triglycerides) and students T test (all other lipids); **p*<0.05, ****p*<0.0001.

There was no difference in the total number of pups born to control and obese dams (Figure 3.4.A). Whereas control dams had pups of an equal sex ratio, obese dams had a significantly higher fraction of male pups (Figure 3.4.B). Obese dams also had pups that were significantly lighter at birth compared to control dams (Figure 3.4.C).

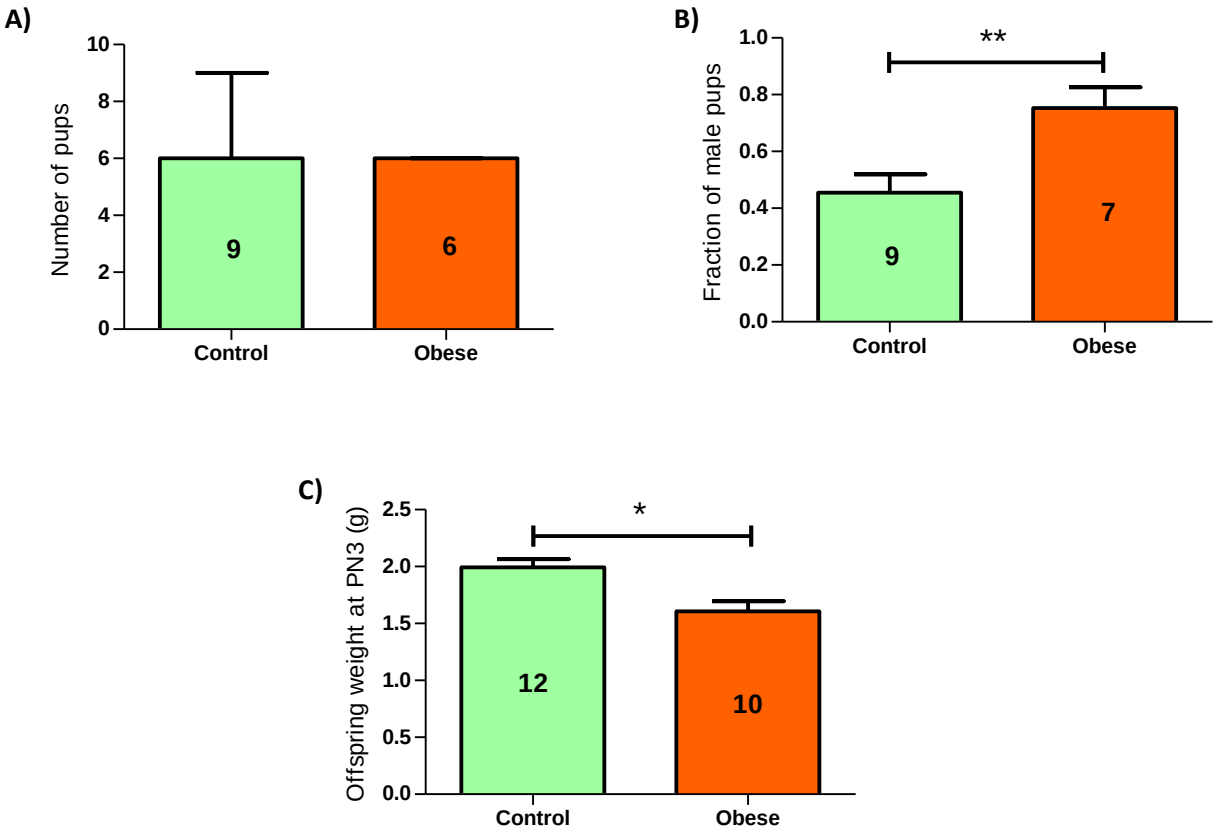


Figure 3.4. **Offspring phenotype at birth. A)** The total number of pups. **B)** The fraction of male pups born**. C)** Offspring weight at PN3**.** N numbers are presented within the graphs. Students T test (fraction of male pups), Mann-Whitney U test (all other graphs); *p<0.05, **p<0.001.

3.5 Discussion

The aim of this study was to characterise the physical and metabolic phenotype of dams fed a highly palatable obesogenic diet from 6 weeks of age and throughout gestation and lactation, and to compare this phenotype to dams fed a control diet throughout the same period.

3.5.1 Dam phenotype

The data presented in this chapter demonstrate that feeding dams a diet rich in fat and sugars (typical of a western diet in humans) from 6 weeks of age and throughout pregnancy and lactation, is sufficient to induce and maintain maternal obesity. This is supported by the fact that obese dams were heavier than control dams at mating, and remained heavier throughout the study period. This increase in weight in obese dams was due to an increase in fat mass. These results are consistent with previous findings from our laboratory (Fernandez-twinn et al. 2012; Blackmore et al. 2015; Fernandez-Twinn et al. 2017), and therefore highlight the reproducibility of the model.

Dam serum wasn't analysed in this study during pregnancy but our laboratory has shown previously that obese dams are glucose intolerant and hyperinsulinaemic on gestational day 18 (Fernandez-Twinn et al. 2017). The results presented here show that whilst insulin levels remain elevated in obese dams following weaning, glucose tolerance returns to normal. This suggests that during pregnancy, which poses an added challenge of exaggerated peripheral insulin resistance, the increase in insulin in obese dams is insufficient to control glucose levels, resulting in gestational diabetes. However, following pregnancy and lactation, hyperinsulinaemia is sufficient to maintain glucose levels in obese dams. These results mirror typical GDM in obese women, where glucose intolerance occurs during the challenge of pregnancy and where only 3-5% of women remain diabetic immediately following pregnancy (Bird et al. 2008).

Dams fed the obesogenic diet also had increased FFA and cholesterol levels following weaning. A previous study using an identical mouse model of maternal obesity also demonstrated that obese dams had increased cholesterol at weaning (Samuelsson et al. 2008). This emphasises the reproducibility of our model. Notably, there is substantial evidence that women with GDM are also highly likely to show postpartum dyslipidaemia (Dwyer et al. 2013; Burlina et al. 2016). Furthermore, raised FFAs are a hallmark of insulin resistance, particularly in obese individuals (Catalano & Hauguel-De Mouzon 2011). These results again highlight our model as an ideal tool for studying the programming effects of maternal obesity with GDM, which is now common among women in the western world.

3.5.2 Potential maternal programming factors

The fact that obese dams showed alterations in several metabolites poses an important question; which of these metabolites could be important programming factors for offspring health? As discussed above, although there was no difference in glucose tolerance at weaning in the current study, previous results from our lab have indicated that obese dams have impaired glucose clearance during pregnancy. The Pedersen hypothesis was formulated to explain fetal macrosomia in response to maternal diabetes. Pedersen reasoned that maternal hyperglycaemia would trigger fetal hyperglycaemia, leading to high insulin release from the fetal pancreas and resulting in greater fetal glucose use and consequently overgrowth (Catalano & Hauguel-De Mouzon 2011). Results from the Hyperglycaemia and Adverse Pregnancy Outcome (HAPO) study in women with GDM supported this hypothesis, demonstrating that increased maternal glucose levels were associated with offspring macrosomia and increased cord blood serum C-peptide levels (an indicator of fetal plasma insulin levels)(The HAPO Study Cooperative Research Group 2008). Furthermore, a mild STZ intervention during pregnancy in rats, resulting in maternal hyperglycaemia, led to macrosomic offspring (Merzouk et al. 2000). Importantly, these offspring showed high adiposity and displayed alterations in glucose and lipid metabolism in adulthood. These studies emphasise that glucose is an important programming factor leading to long term adverse outcomes in offspring.

In the current study, offspring exposed to maternal obesity were lighter at day 3 than offspring exposed to a maternal control diet. This is in contrast to the studies discussed above where macrosomia was observed in offspring exposed to maternal obesity, and goes against the Pedersen hypothesis. However, macrosomia may not always result from exposure to maternal hyperglycaemia. Numerous studies utilising STZ to induce maternal hyperglycaemia in rodents demonstrate a reduction in offspring birth weight (Tran et al. 2008; Chen et al. 2010; Hokke et al. 2013; Aliou et al. 2016). Furthermore, rat offspring exposed to a maternal high fat and fructose diet, similar to our model, were also lighter at birth compared to control offspring (Yamada-Obara et al. 2016). These animals went on to develop metabolic, cardiovascular and renal impairments later on in life, highlighting that programming through early life high glucose exposure may occur despite a lack of fetal overgrowth. Unpublished data from our laboratory has also shown that at embryonic day 19, pancreatic insulin content is increased in offspring of obese dams. This highlights a detachment of maternal hyperglycaemia leading to fetal hyperinsulinaemia, and offspring growth. As discussed above, exposure to maternal obesity or GDM often leads to an increase in adiposity in offspring. Body composition was not determined at birth in the present study, however another study where offspring were exposed to a maternal western diet showed that offspring had increased fat mass at birth despite showing no alteration in weight (Frihauf et al. 2016). This again emphasises that maternal

hyperglycaemia may program the offspring metabolism without increasing birth weight and highlights that glucose could be a relevant programming factor for offspring outcomes in this PhD. Finally, data from humans has shown that maternal obesity is a major risk factor for SGA offspring (McCowan et al. 2013) as well as LGA offspring, again highlighting our model as a translatable tool for studying the programming effects of maternal obesity.

As mentioned above, previous studies from our laboratory have shown that obese dams are hyperinsulinaemic during pregnancy, and the current study showed that obese dams were also hyperinsulinaemic at weaning. Hyperinsulinaemia in pregnancy is associated with exaggerated insulin resistance. Maternal insulin resistance increases fetal macronutrient uptake, including glucose (Brett et al. 2014). As per the Pedersen hypothesis, increased fetal glucose uptake leads to fetal hyperinsulinaemia. As mentioned above, we have seen high pancreatic insulin levels in E19 fetuses exposed to maternal obesity, consistent with the notion that maternal hyperinsulinaemia could be an important programming factor in our model. Importantly, a study utilising female mice haplo-insufficient for insulin receptor substrate 1 (IRS-1) and demonstrating hyperinsulinaemia and insulin resistance, but normal glucose tolerance and body weight, showed that exposed male offspring were hyperinsulinaemic and glucose intolerant by 1 month of age and had increased lipids within the liver by 6 months of age (Isganaitis et al. 2014). This study emphasises that increased maternal insulin levels *per se,* independent of other metabolic abnormalities, can have drastic implications for offspring metabolism, and suggests that maternal insulin could play an important role in offspring metabolic programming in the current study.

Insulin resistance in obese women with GDM is associated with dyslipidaemia (Catalano & Hauguel-De Mouzon 2011). Accordingly, obese dams showed increased cholesterol and FFA levels in the current study. There is ample evidence suggesting that excess lipid availability to the fetus plays an important role in fetal programming, particularly fetal adiposity (Catalano & Hauguel-De Mouzon 2011). Fetal cholesterol levels correlate with maternal cholesterol levels (Napoli et al. 1997). The fetus utilises cholesterol to produce steroid hormones (Ghio et al. 2011). Steroids are known to vastly effect offspring development (see chapter 1, section 1.5.2.2), suggesting maternal cholesterol levels could be an important factor effecting the offspring phenotype. It has also been demonstrated in humans that fetuses exposed to maternal hypercholesterolaemia develop fatty streaks within the aorta *in utero* (Napoli et al. 1997). This highlights maternal cholesterol as an important programming factor influencing the offspring cardiovascular system, with potential implications for later life CVD. In contrast to humans, rodents are resistant to the development of atherosclerosis. Nevertheless, the above evidence highlights maternal cholesterol as a potential programming factor associated with maternal obesity in the current study.

Increased FFAs are a hallmark of insulin resistance, especially in obese individuals (Catalano & Hauguel-De Mouzon 2011). Maternal serum FFA levels have been shown to correlate with neonatal abdominal circumference and offspring adiposity at birth (Schaefer-Graf et al. 2008), suggesting FFAs may be influential for the offspring phenotype, particularly offspring metabolism. Maternal FFAs present another possible variable leading to changes in the offspring phenotype in the current study. In the future, determining fetal and neonatal body composition might provide more insight into whether maternal FFAs are important for offspring programming in our model. To date, this has not been possible due to the technical difficulties associated with performing body composition analysis in young mice.

Our model encapsulates maternal obesity and GDM in western women, making it a useful research tool. However, the multitude of potential programing characteristics that come with maternal obesity present a challenge. Teasing apart which of these maternal characteristics might be important for health outcomes in offspring is crucial if we are to develop effective interventions to prevent ill health in future generations. This will be addressed more in Chapter 7.

3.5.3 Litter phenotype

Although there was no difference in the total number of pups born to obese and control dams, obese dams had more male than female offspring. Studies have shown that rodents typically produce equal numbers of male and female pups when conditions are optimal (Schlager & Roderick 1968; Labov et al. 1986). The Trivers and Willard hypothesis states that in polygynous species such as mice, where a small number of the fittest males produce most of the offspring, females in good condition relative to other females in the population are more likely to produce male progeny, since these males are likely to be healthy and have a high chance of siring lots of offspring in the future. Conversely, females in a poor condition are expected to produce more females, since the ability to reproduce in females is not as dependent on body condition as in males (Trivers & Willard 1973). In support of this hypothesis, rodent models of maternal food restriction (Meikle & Drickamer 1986; Labov et al. 1986; Wright et al. 1988), protein restriction (Kwong et al. 2000) and fatty acid restriction (Rivers & Crawford 1974), have demonstrated a skew towards female offspring. Furthermore, mouse dams fed a high fat diet and thus exposed to abundant energy availability, were significantly more likely to produce male offspring (Rosenfeld et al. 2003). This phenomenon has been observed in other polygynous mammals too. Studies in different species of deer have demonstrated that mothers fed a high energy diet, or mothers in good body condition, had more male offspring (Wauters et al. 1995; Flint et al. 1997; Enright et al. 2001). This evidence suggests that obese dams in the current study could have produced more males

due to an abundance of energy available from the obesogenic diet. However, although dams on the obesogenic diet consumed more energy than control dams throughout gestation and lactation, it is unlikely that this extra energy was available for offspring development *in utero*. Offspring born to obese dams in this study were lighter at birth compared with offspring of control dams, consistent with growth restriction seen in animal models of maternal nutrient restriction or placental insufficiency and other models of maternal over-nutrition. This either suggests that the skew towards male offspring in obese dams seen in the current study was not due to deliberate allocation, or that obese dams wrongly predicted that the abundant resources available to them would also be available to their offspring *in utero*. However in the longer term, male pups from obese dams put on weight faster during lactation and were heavier than male pups from control dams by weaning (see chapter 4), showing that abundant resources were available to obese dams and pups during lactation. This is therefore consistent with the rationale of increasing resource allocation to male fetuses and increasing the number of male pups in the litter as observed.

In support of this, another plausible reason for unequal sex allocation in differing environmental conditions is sexual dimorphism, where one sex is larger than the other and therefore requires more resources for growth. Studies in birds have shown that when resources are abundant, females have more offspring of the larger, dominant sex, since this sex requires more care and energy during early life (Komdeur 2012). It is possible then, that the obese dams in this study had more males since, in mice males are the larger dominant sex and, obese dams had plentiful food available for lactation.

Finally, it should be noted that litters with an unequal sex ratio can occur spontaneously in rodents (Rosenfeld et al. 2003), highlighting the possibility that the skew towards male pups in obese dams in the current study was due to chance. It will therefore be important to repeat this study to confirm the observation.

3.5.4 Limitations and future directions

One limitation of this study was that metabolic parameters were not measured during pregnancy. Conducting these measures would have introduced another variable that may have confounded programming effects in offspring. However, studies from our laboratory using a separate cohort of dams that were culled on day 19 of pregnancy have demonstrated that obese dams are glucose intolerant at gestational day 18. Since our model has proven to be highly reproducible in the past we can assume that the dams in this study had impaired glucose tolerance and were hyperinsulinaemic during pregnancy. Another limitation of this study was that, due to associated technical challenges, neonatal body composition was not assessed. Determining fat mass in offspring of obese dams might

provide more insight into the potential impacts of maternal hyperinsulinaemia, hyperglycaemia and hyperlipidaemia.

3.5.5 Conclusions

The results of this study have demonstrated that feeding a highly palatable diet, rich in fat and sugars and typical of the western diet, is sufficient to induce obesity in female mice. Obese dams were heavier than control dams and showed a significant increase in fat mass throughout the study period. These changes in body composition were also accompanied by markers of metabolic dysfunction. The maternal characteristics observed in this study are consistent with those seen in obese women with GDM, including hyperinsulinaemia, hypercholesterolaemia and increased FFAs thus emphasising our model as a translatable tool for studying the programming effects of maternal obesity. Going forward, it will be important to address the question of which maternal characteristics are important for offspring programming, so that effective interventions that prevent poor health in future generations can be established.

3.5.6 Summary

- Maternal obesity can be successfully induced in C57BL6 mice by feeding a highly palatable obesogenic diet, rich in fat and sugar, from 6 weeks of age.
- Dams on the obesogenic diet show an increase in body weight which is due to an increase in fat mass and not lean mass.
- At weaning, obese dams demonstrate hyperinsulinaemia and dyslipidaemia but show normal glucose levels.
- A number of metabolic and physical parameters associated with maternal obesity during pregnancy could mediate the detrimental effects in the offspring.

Chapter 4. Maternal obesity alters offspring renal morphology at weaning

4.1 Introduction

4.1.1 The CKD epidemic – a role for the early life environment

As discussed in chapter 1, section 1.1, CKD is increasing globally at alarming rates concurrently with obesity. Hypertension and diabetes contribute to a large proportion of CKD cases but many remain unexplained. The quality of the intrauterine environment is known to influence organ development and is a likely, albeit less recognised cause of later life CKD (Ritz et al. 2011).

4.1.1.2 Maternal diabetes/gestational diabetes

The suggestion that the kidney is susceptible to early life environmental influences is supported by epidemiological studies of maternal diabetes. Pre-gestational maternal diabetes correlates with kidney and urinary tract malformations in exposed offspring more so than cardiovascular congenital malformations or multiple congenital malformations (Nielsen et al. 2005; Davis et al. 2010; Khalil et al. 2010; Hsu et al. 2014). Similarly to studies of under-nutrition (see chapter 1, section 1.4.1), exposure to maternal diabetes appears to lead to poor renal outcomes. A study of Pima Indians (a population with one of the highest rates of diabetes in the world) demonstrated that individuals whose mothers were diabetic during pregnancy were 3.8 times more likely to develop albuminuria than their siblings born before the onset of maternal diabetes (Nelson et al. 1998). This observation emphasises the importance of the early life environment for renal health independent of genetic factors.

4.1.1.3 Maternal obesity

To date, only one epidemiological study has assessed the impact of maternal obesity on the offspring kidney. Hsu and colleagues showed that increased maternal BMI, independent of maternal smoking, hypertension and GDM was associated with renal malformations in children including dysplasia/aplasia and obstructive uropathy (Hsu et al. 2014). This suggests that maternal obesity could be an important renal programming factor. However the impact of maternal obesity on offspring renal health remains under-explored and poorly defined. This might be partly due to the fact that the obesity epidemic is fairly recent, and human lifespans are long, meaning there simply hasn't been sufficient time to collect information relating to effects of maternal obesity on offspring CKD. Furthermore, CKD has associated co-morbidities including CVD and diabetes; with CKD sufferers most likely to die from CVD. As such, studies focusing on the impact of maternal obesity may have

concentrated on CVD and diabetes as outcomes, overlooking potential programming of the kidney or CKD. Therefore, there is little to no information on the potential contribution of early life obesity exposure on the current CKD epidemic.

4.1.2 Animal models support the idea that the kidney is susceptible to early life programming

The effects on the offspring kidney resulting from different adverse maternal environments described in this section are summarised in Table 1.2, chapter 1.

4.1.2.2 Animal models of maternal diabetes

Many rodent models have assessed the impact of maternal diabetes on offspring renal morphology and have shown either a reduction in nephron number (Nehiri et al. 2008; Tran et al. 2008; Hokke et al. 2013), or glomerular hypertrophy (Rocha et al. 2005; Magaton et al. 2007; Rocco et al. 2008). These two factors are often inter-related but can occur independently (see section 4.5.3). Offspring exposed to maternal diabetes go on to develop indicators of renal dysfunction including proteinuria (Nehiri et al. 2008; Chen et al. 2010; Aliou et al. 2016) and a reduction in GFR (Rocha et al. 2005; Magaton et al. 2007; Rocco et al. 2008), highlighting an association between adverse renal morphology and later life renal disease. These studies suggest that maternal obesity or GDM in humans could contribute to the current CKD epidemic. However, it can be difficult to compare animal models of maternal diabetes with human GDM as the nature and development of diabetes in these models often do not reflect the human situation. In the models mentioned above, diabetes was induced in dams by the administration of STZ which leads to hyperglycaemia through the rapid ablation of pancreatic beta cells and inhibition of insulin production (King 2012). In contrast GDM in humans is characterised by pancreatic beta cells being unable to compensate for peripheral insulin resistance (Kühl 1991). Models using STZ therefore mimic insulin deficiency that occurs in type 1 diabetes, whereas human GDM is characterised by insulin resistance in the face of hyperglycaemia and is more similar to type 2 diabetes. Furthermore, STZ is toxic to other organs in the body as well as the pancreas (King 2012) which limits the translatability of such models for the study of offspring outcomes following GDM.

4.1.2.3 Animal models of maternal high fat diets

In contrast to models of maternal diabetes, models of maternal high fat diet do not show renal morphology adversities in offspring. Rats exposed to a maternal diet rich in lard showed no differences in renal weight, nephron number or glomerular volume (Armitage et al. 2005). Remarkably, mice exposed to a maternal high fat diet during gestation and lactation had 20-25% more nephrons by

gestational day 18.5 and by weaning when compared to control mice, and displayed normal renal morphology and function in adulthood (Hokke et al. 2016) Mothers of offspring exhibiting this phenotype had normal weights and glucose profiles, supporting the notion that weight and glucose intolerance in pregnancy may be important renal programming factors.

4.1.2.4 Animal models of maternal obesity

Only a couple of studies have investigated the impact of a maternal obesogenic diet on offspring renal health. Jackson and colleagues showed that male rats exposed to a maternal high fructose and high fat diet (resulting in maternal obesity) during pregnancy and lactation had increased albumin excretion, glomerulosclerosis and tubulointerstitial fibrosis (Jackson et al. 2012). Furthermore, female mice exposed to a maternal high fat/fructose diet showed a decrease in GFR (Flynn et al. 2013). These observations emphasise the potential role for a maternal obesogenic diet for the programming of later life renal health.

4.1.2.5 The value of a maternal diet induced obesity model for renal studies

The CKD epidemic is rising rapidly and is concurrent with increasing obesity in the population including during pregnancy and lactation. It is clear from both epidemiological and experimental studies that renal morphology and health is influenced by the early life environment. Despite these observations, the impact of maternal diet-induced obesity upon offspring renal morphology has not been investigated. The aim of this chapter was to characterise renal morphology in 3 week old mice exposed to maternal diet-induced obesity throughout gestation and lactation. As nephrogenesis continues for 1 – 2 weeks following birth in mice, the 3 week time point was selected since by this time nephrogenesis should be complete, yet nephron loss due to ageing should not have started. Characterising early life renal morphology in offspring exposed to maternal obesity might shed light on why these offspring are susceptible to poor renal health in adulthood. Since our model of maternal obesity is similar to maternal obesity in humans, findings may ultimately provide information that could help in the prevention of CKDs in the future.

4.2 Aims

The aims of this chapter were:

1. To characterise the phenotype of offspring exposed to either an obesogenic environment or control environment throughout gestation and lactation; from birth until weaning.
2. To determine the metabolic profile of offspring at weaning.
3. To characterise renal morphology in offspring at weaning.

4.3 Methods

4.3.1 Offspring phenotyping

4.3.1.1 Offspring weight and organ weights

Litters from control and obese dams were weighed on postnatal (PN)3, PN7, PN14 and PN21. The offspring growth trajectory was calculated as the average littermate weight per litter (mixed sex). Following an overnight fast at weaning (PN21), 1 male per litter was sacrificed by rising CO_2 asphyxiation. The organs were collected and weighed.

4.3.1.2 Serology

Tail blood glucose measures were conducted as described in chapter 2, section 2.2. Serum was collected following an overnight fast as described in chapter 2, section 2.3.1. Serum was analysed by the mouse biochemistry phenotyping facility (Institute of Metabolic Science) for cholesterol (LDL and HDL), triglycerides, insulin, leptin and corticosterone. Haemolysed samples were excluded. Cholesterol, triglycerides, insulin and leptin were measured as described in chapter 2, section 2.3.2. Corticosterone was measured by enzyme immunoassay (Immunodiagnosticsystems, Tyne & Wear, UK). The lower limit of detection was 17ng/ml. The intra coefficient of variance for the assay was 3.5%.

4.3.2 Renal morphology

4.3.2.1 Sectioning and staining

Kidneys were post-fixed in formalin from 3 week males and were transferred to 70% ethanol for storage. When required, kidneys were processed and embedded into a paraffin block and were exhaustively sectioned at 5µm. Sections were then stained with Haematoxylin and Eosin. Sectioning and staining was performed by the Institute of Metabolic Science histology core at Addenbrookes Hospital who coded the sections so that the student was blind to maternal dietary groups when analysing the sections. High resolution (226nm/pixel) images were taken of the sections using a nanozoomer (NDP. Scan 2.5.88, Hamamatsu Photonics UK Limited).

4.3.2.2 Measuring medulla and cortex areas

One section was used for medulla and cortex measurements every 30 sections throughout the entire kidney. Sections were visualised using an Olympus U-PMTVC (8H01 420) microscope fitted with a motorised specimen stage and microcator. Quantitative analyses were performed using the Computer Assisted Stereology Toolbox (CAST) version 2.0 program (Olympus, Denmark). A grid was

superimposed onto each kidney section at a magnification of 1.25x. Any points superimposed on the medulla and cortex areas of the sections were counted. The mean medulla and cortex area for each kidney was then calculated as follows;

Mean number of points per section x area per point

The total medulla and cortex area counted throughout each kidney was used to calculate glomeruli densities (number glomeruli/mm^2) since glomeruli are only found in these areas.

4.3.2.3 Estimating total nephron number and nephron density.

Section pairs were used to count nephrons using the physical dissector/fractionator combination, the "gold standard" method for estimating nephron endowment. Section pairs were taken every 30 sections, this is recommended in postnatal mouse kidneys in order to avoid counting the same nephrons twice (Cullen-McEwen et al. 2011). The student was blinded to study groups to avoid experimenter bias. Using NDP view 2 (Hamamatsu Photonics UK Limited), the entire 1st section (n) was magnified at 4x on a computer screen and all the visible nephrons were marked on acetate overlaying the image. The n+2 section was then magnified at 4x and the same acetate was used to count all nephrons that were on section n but no longer on section n+2. Nephrons present on section n+2 but not on section n were also counted (Figure 4.1). This technique was repeated for each pair of sections throughout the entire kidney (>10 pairs were counted for each kidney for reliable measures). The total number of nephrons was then calculated in each kidney as follows:

$$\boldsymbol{N}\mathbf{glom} = 30 * ½ * ½ * \boldsymbol{Q}^{-}$$

Where ***N*glom** is the estimated total number of nephrons in the kidney. **30** is the number of sections advanced between section pairs. The first **½** accounts for the fact that the dissector pair of sections consisted of the n and the n+2 sections. The last **½** accounts for the fact that nephrons were counted in both directions between the two sections of a pair. $\boldsymbol{Q}^{-}$ is the actual number of nephrons appearing and disappearing between n and n+2 sections.

The mean glomeruli density in each kidney was calculated as follows:

$$\textbf{Glomeruli denisity} = \mathbf{Q}^{-} / \textbf{section area}$$

Where $\mathbf{Q}^{-}$ is the total number of nephrons appearing and disappearing between n and n+2 sections counted in all pairs of sections (Figure 4.1), and **section area** refers to the total medulla and cortex area of all n+2 sections.

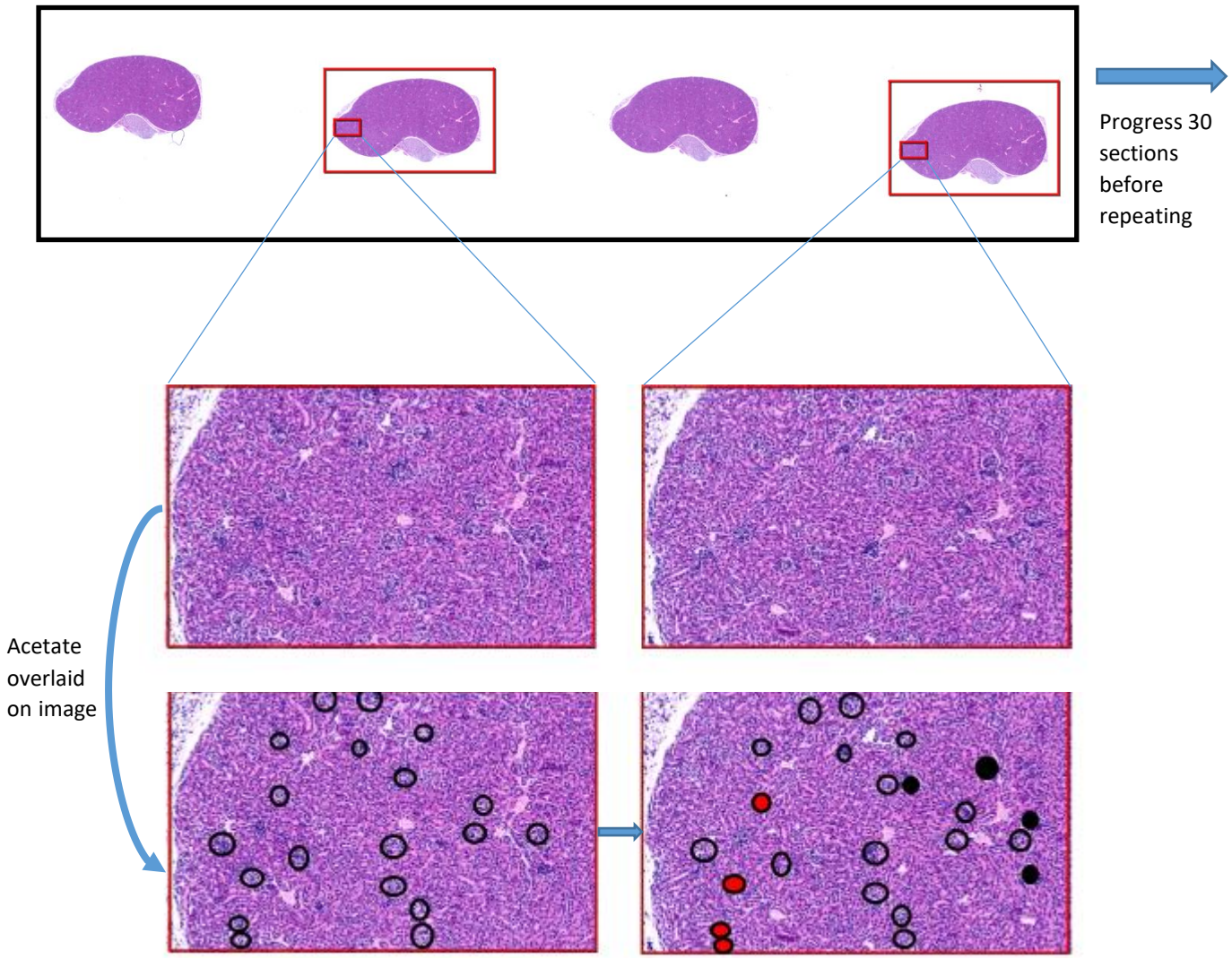


Figure 4.1. **Method of nephron counting in 3 week male kidneys.** Using clear acetate, glomeruli in section n were drawn around in black. This same acetate was then overlaid on the n+2 section and disappeared glomeruli (those present on n but not on n+2) were coloured in red. Appeared glomeruli (those present on n+2 but not on n) were drawn around and coloured in black.

4.2.2.4 Estimating glomeruli diameters

Around 30 sections into each kidney (150µm) a random section was selected for glomeruli width calculation. The glomeruli on this section were known to be representative of glomeruli throughout the whole kidney due to glomeruli counting studies that had been performed previously. Using NDP view 2, the section was magnified at 7x and the longest possible glomerular tuft diameter was measured for all visible glomeruli in that section (Figure 4.2). Over 190 glomeruli were measured in each kidney. Again, the researcher was blinded to study groups.

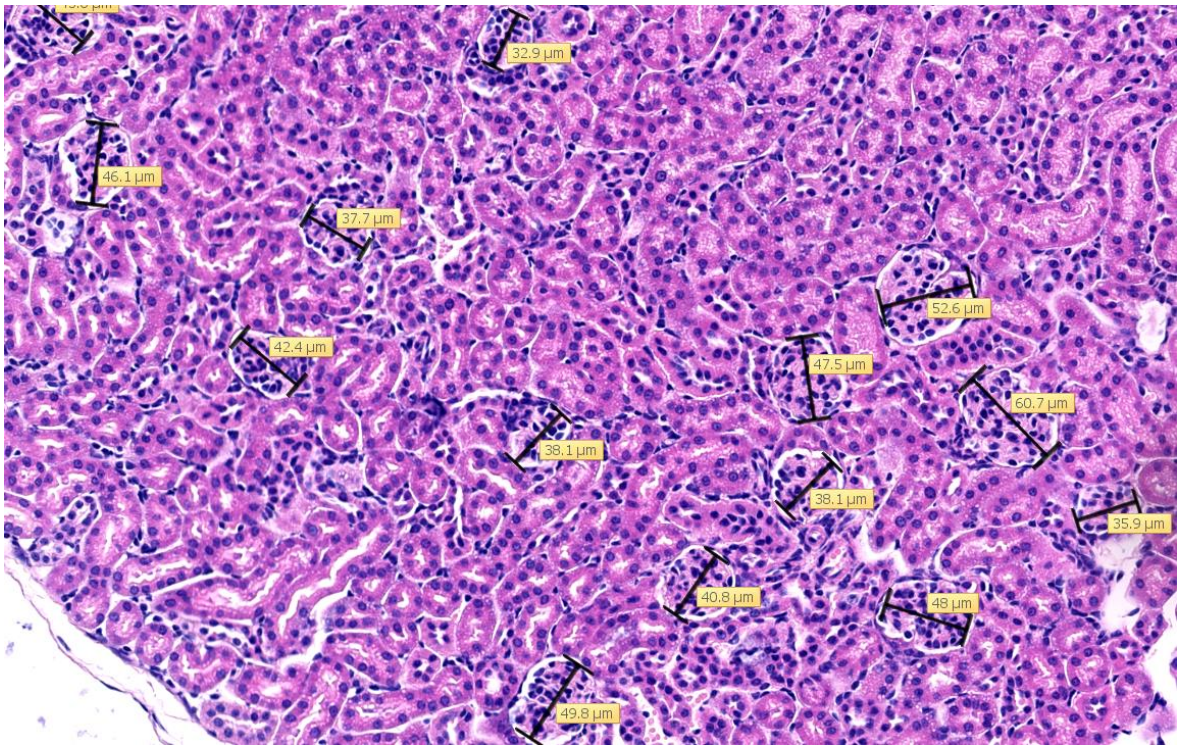


Figure 4.2. **Method of measuring glomeruli diameters.** NDP view 2 was used to magnify images. A ruler function was then used to measure the longest possible diameter of each glomerular tuft.

4.3.3 Statistics

All statistical analyses were conducted using Prism 5.0 (GraphPad, CA, USA). All data were checked for normality by either a Shapiro-Wilk or a Kolmogorov–Smirnov (for smaller data sets) test. Non-normal data were analysed using Mann-Whitney U tests and the median and IQR are presented. Normal data were analysed by students T tests and the mean and SEM are presented. For all data sets, $p<0.05$ was considered statistically significant. Sample sizes (n) are reported within each figure, where n represents the number of independent litters per experimental group. Importantly, except for the body weight trajectory, only 1 male per litter at each time point was used to avoid litter bias.

4.4. Results

On PN3, offspring exposed to maternal obesity were significantly lighter than offspring exposed to a maternal chow diet (Figure 4.3.A). By PN7 the body weight of the offspring had caught up with that of controls hence on PN7 and PN14 maternal diet had no significant effect on offspring weight (Figure 4.3.A). However, by PN21 offspring exposed to maternal obesity showed a significant increase in body weight (Figure 4.3.A). Offspring of obese dams remained significantly heavier than offspring of control dams following an overnight fast at weaning (Figure 4.3.B).

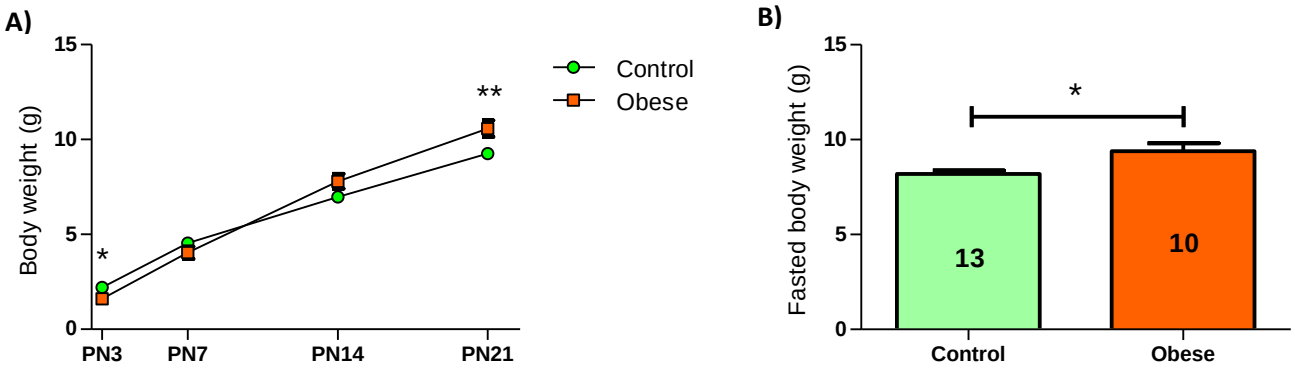


Figure 4.3. **Offspring body weight up to 3 weeks of age. A)** Offspring body weight from PN3 to PN21. Mann-Whitney U test (PN3 & PN21), students T test (PN7 & PN14). N= 13 control, 10 obese. **B)** Offspring body weight following an overnight fast at weaning. Students T test. N numbers are presented within the graph. *p< 0.05, ** p< 0.001.

At weaning, offspring heart weight was significantly increased by exposure to maternal obesity. This association remained significant when heart weight was expressed relative to body weight. Kidneys and liver weights of offspring exposed to maternal obesity were also significantly heavier than those of control offspring. However, when normalised to body weight, maternal diet had no significant effect on kidney and liver weight. Exposure to maternal obesity also significantly increased offspring subcutaneous, epididymal and retroperitoneal fat pad mass. These associations were maintained after normalising fat pad mass to body weight. There was no effect of maternal diet on offspring vastus weight or vastus normalised to body weight. These results are summarised in Table 4.1.

Table 4.1. *Offspring organ weights at 3 weeks of age.*

Absolute organ weights (mg)	**Control (n=12)**	**Obese (n=14)**
Heart	67.1±1	102.5±4.9***
Total kidneys	124±4	145±6*
Liver	293±10	366±17**
Subcutaneous fat	42.1±3.1	124.7±17.9***
Epididymal fat	10.8±1.3	46.4±7.9***
Retroperitoneal fat	3±5.8	13±33**
Vastus	63.76±2.9	69.36±4.46
Organ weights (% of body weight)	**Control (n=12)**	**Obese (n=14)**
Heart	0.82±0.03	1.05±0.04***
Total kidneys	1.54±0.42	1.51±0.12
Liver	3.76±0.14	3.88±0.11
Subcutaneous fat	0.52±0.04	1.31±0.17***
Epididymal fat	0.13±0.01	0.55±0.09***
Retroperitoneal fat	0.03±0.07	0.13±0.06*
Vastus	0.79±0.04	0.74±0.03

N numbers are presented within the table. Mann-Whitney U test (retroperitoneal fat, total kidney [% body weight], retroperitoneal fat [% body weight]), Students T test (all other factors); *p<0.05, **p<0.001, ***p<0.0001.

At weaning, offspring fasting serum total cholesterol and triglycerides were significantly increased by exposure to maternal obesity. There was no significant effect of maternal diet on offspring FFA. Insulin and glucose levels were also significantly increased by exposure to maternal obesity. Maternal obesity significantly decreased offspring serum corticosterone levels. These results are summarised in Table 4.2.

Table 4.2. *Offspring fasting serology measures at 3 weeks of age.*

	Control (n=10)	**Obese (n=10)**
Total cholesterol (mmol/l)	2.31±0.09	2.62±0.40*
Triglycerides (mmol/l)	0.78±0.10	1.20±0.07**
FFA (µmol/l)	947±73	813±87
Insulin (pmol/l)	21±4.9	48±7.7*
Blood glucose (mmol/l)	6.9±0.4	9.2±0.9*
Corticosterone (ng/ml)	480±53.6	319±35*

N numbers are presented within the table. Students T test; *p<0.05, **p<0.001.

The combined medulla and cortex area was significantly increased in offspring exposed to maternal obesity (Figure 4.4.A) and this was due to a significant increase in cortex area (Figure 4.4.A). Glomeruli density was significantly decreased by exposure to a maternal obesogenic diet (Figure 4.4.B). However, there was no difference in total glomeruli number within the kidneys of offspring exposed to maternal obesity compared to those of control dams (Figure 4.4.C). Maternal diet also had no significant effect on offspring total glomeruli number normalised to body weight (Figure 4.4.D). However, exposure to maternal obesity did significantly increase offspring glomeruli diameters (Figure 4.4.E).

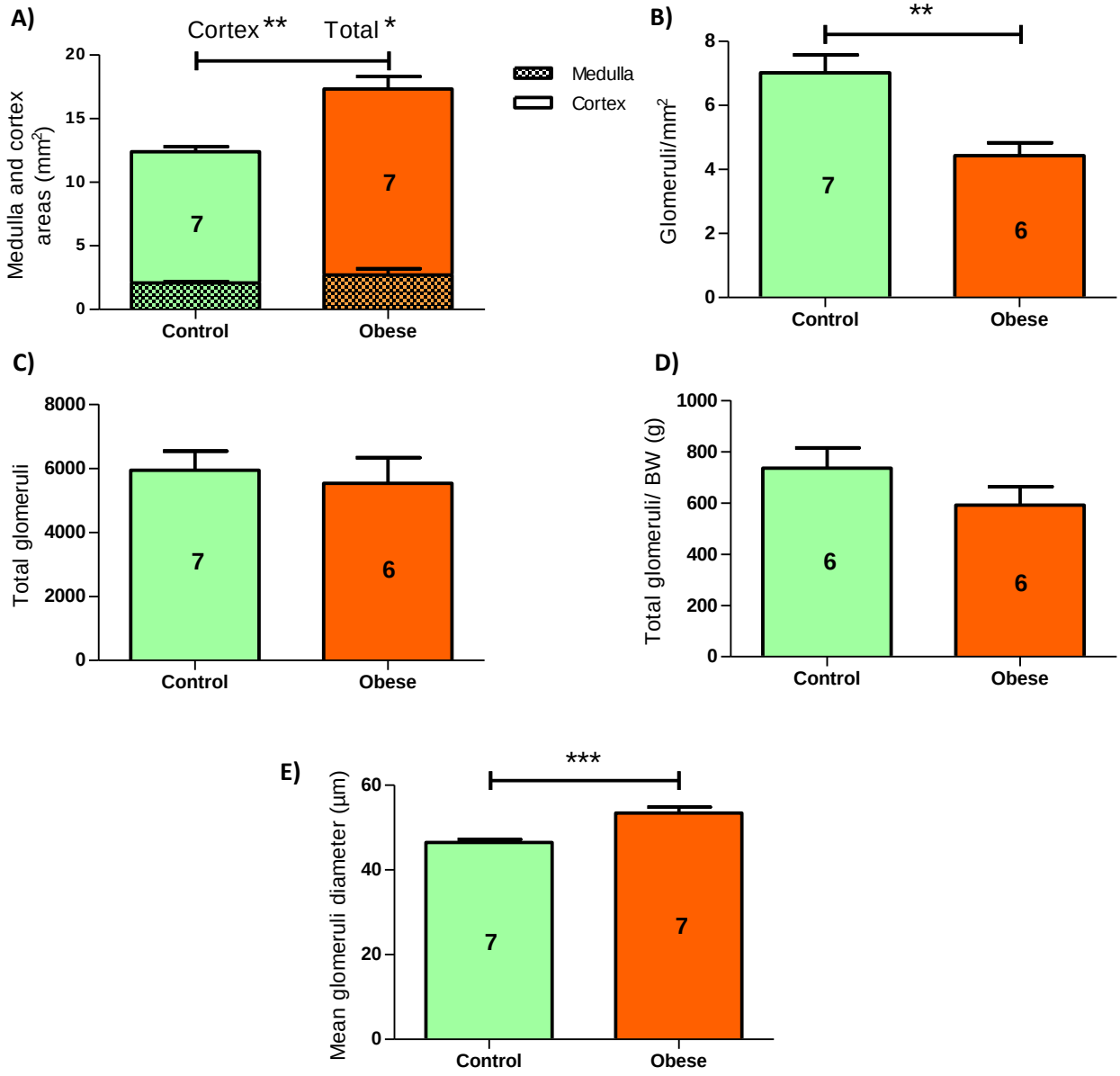


Figure 4.4. **Renal morphology in 3 week old offspring. A)** Medulla and cortex areas. **B)** Glomeruli density. **C)** Total glomeruli number. **D)** Total glomeruli number normalised to body weight. **E)** Glomeruli diameter. N numbers are presented within the graphs. Mann-Whitney U test (glomeruli density), Students T test (all other graphs); *p< 0.05, **p<0.001, *** p< 0.0001.

4.5 Discussion

The aim of this chapter was to characterise the physical, metabolic and renal morphological phenotype of offspring at weaning exposed to a maternal obesogenic diet, and to compare this phenotype to offspring exposed to a maternal control diet.

4.5.1 Physical phenotype

The data presented in this chapter demonstrate that offspring of obese dams were growth restricted at birth but underwent postnatal catch up growth during lactation and were heavier than control offspring by weaning. In humans postnatal catch up growth has been associated with later life CVD (Eriksson et al. 1999), whilst in animal models catch up growth has been shown to accelerate ageing (Jennings et al. 1999; Tarry-Adkins et al. 2009), increase serum glucose levels (Martin-Gronert et al. 2008) and impair renal health (Boubred et al. 2009; Chen et al. 2010)(discussed in Chapter 1, section 1.5.1.3). These studies suggest that exposure to maternal obesity could lead to poor health later in life by means of promoting growth restriction and catch up growth.

Consistent with previous findings in our laboratory, offspring of obese dams showed an increased absolute heart weight and heart weight normalised to body weight. We have shown previously that 8 week old male mice exposed to maternal obesity had an increased heart mass and this was associated with left ventricular wall thickening and cardiomyocyte hypertrophy (Fernandez-twinn et al. 2012). Importantly, these offspring also demonstrated cardiac dysfunction by 12 weeks of age (Blackmore et al. 2015). The increased heart weight in offspring of obese dams in the current study therefore suggests that offspring may show changes in cardiac morphology from a very early age and that, consistent with our previous cohorts, these offspring may be more at risk of cardiac dysfunction.

Offspring exposed to maternal obesity also showed increases in absolute fat pad mass, and normalised to body weight at weaning. This suggests that the overall increase in body weight at weaning in offspring of obese dams was largely due to an increase in fat and not lean mass. Rats exposed to a maternal western diet also showed increased adiposity at 3 weeks of age (Frihauf et al. 2016). Additionally, in humans increased childhood adiposity has also been observed in association with maternal GDM (Lawlor et al. 2010), GWG (Mamun et al. 2009) and BMI (Catalano et al. 2009). These studies together with the current data emphasise that maternal over-nutrition is a promoter of offspring adiposity at a young age. In individuals exposed to high GWG, the increased adiposity phenotype persisted into adulthood (Mamun et al. 2009). Accordingly, high adiposity in childhood has been shown to be a predictor of obesity in adulthood, as well as T2DM, hypertension, dyslipidaemia,

and atherosclerosis (Juonala et al. 2011). The increased adiposity in 3 week old offspring exposed to maternal obesity in the current study is therefore another factor suggesting that these offspring may be more at risk of later life cardiometabolic disease.

4.5.2 Metabolic phenotype

Offspring of obese dams also showed increased fasting serum cholesterol, triglycerides, insulin and glucose levels. It is known that during pregnancy, maternal visceral fat, insulin resistance, lipids and triglycerides levels increase. However during lactation, lipids are transferred to milk to shift resource allocation to the growing infant (Stuebe & Rich-Edwards 2009). Since obese dams showed higher insulin and cholesterol levels just after weaning, it is likely that obese dams also showed an alteration in milk composition. Exposure to energy rich milk throughout lactation is one plausible explanation for the adverse serum metabolic profile seen in offspring of obese dams at the end of weaning.

The altered serum profile in offspring of obese dams is consistent with the increased fat pad mass also seen in these offspring at weaning. As discussed in Chapter 3, hyperinsulinaemia promotes growth and lipid deposition as per the Pedersen hypothesis, and lipid availability is also known to be a key programming factor affecting offspring adiposity (Catalano & Hauguel-De Mouzon 2011). A previous study using an identical mouse model of maternal obesity demonstrated that offspring had increased adiposity together with hyperinsulinaemia in adulthood (Samuelsson et al. 2008), highlighting the reproducibility of the model. The current data builds on this observation and demonstrates that an increased adiposity phenotype is present in offspring exposed to maternal obesity from a very early age. Furthermore, in humans the relationship between maternal BMI and offspring obesity together with glucose intolerance and dyslipidaemia is well described (Catalano et al. 2009). This emphasises our model as a translatable tool for studying effects of maternal obesity on offspring health. Importantly, high fasting glucose and insulin in children predicts higher glucose, insulin, insulin resistance, blood pressure, lipids and intima-media thickness in adulthood (Yajnik et al. 2015). Again, this emphasises that by weaning, offspring of obese dams already show signs that they may be more at risk of later life health complications associated with metabolic dysfunction.

Offspring exposed to maternal obesity showed a significant decrease in serum corticosterone levels. Blood was collected for corticosterone analysis at post-mortem following an overnight fast. Fasting is known to lead to hypoglycaemia and could lead to stress. The changed corticosterone levels in mice of obese dams suggest that these animals may have altered HPA axis activity and an altered stress response. In a rat model of maternal high fat diet, offspring demonstrated a blunted adrenocorticotropic hormone (ACTH) and corticosterone response to stress during lactation but an

increased ACTH response later on in life (Trottier et al. 1998). The authors showed that high fat feeding increased fat content in the milk, suggesting that exposure to fat alters HPA development and stress reactivity. It has also been suggested that a blunted corticosterone response can occur in response to a novel stressor such as fasting within the setting of chronic stress (Kim et al. 2015). Furthermore, in either dexamethasone exposed animals who exhibit a stressed phenotype (Flagel et al. 2002) or genetic strains that are prone to stress and anxiety like behaviours (Sotnikov et al. 2014), a blunted corticosterone response has been observed to novel stress, highlighting that behaviour indicative of stress is not always associated with increased corticosterone release. In animal studies of postnatal overexposure to glucocorticoids, anxiety like phenotypes have been associated with both increases and blunted corticosterone responses. These studies suggest that positive and negative changes in corticosterone levels indicate altered HPA function and hyper-responsiveness to stress. Therefore, it's possible that offspring exposed to maternal obesity in the current study exhibit altered HPA axis function and are predisposed to stress.

An alternative explanation for the decrease in corticosterone levels in offspring exposed to maternal obesity could be related to the accelerated post-natal catch up growth displayed by these mice. During post-natal development, there is a natural surge in cortisol/corticosterone. In C57BL mice, it has been shown that levels increase from post-natal day 12 and peak in 18-20 day old mice before decreasing back to day 12 levels by post-natal day 30 (Diez et al. 1976). The rate of post-natal growth has been shown to alter the timing of the corticosterone surge. IUGR mice prevented from undergoing catch up growth during lactation showed significantly higher corticosterone levels at 3 weeks of age compared to mice that underwent catch-up growth (Isganaitis et al. 2009). It's therefore possible that offspring exposed to maternal obesity in the current study showed an early decrease in corticosterone levels consistent with accelerated post-natal catch up growth.

4.5.3 Kidney phenotype

By weaning, offspring of obese dams showed an increased kidney area and increased kidney cortex area when compared with offspring of control dams. This was consistent with the increased absolute kidney weight also seen in offspring exposed to maternal obesity. Since there was no difference in kidney weight normalised to body weight between the two offspring groups, it is likely that the increased kidney area in offspring of obese dams reflects growth of the kidney in accordance with overall growth of the offspring during lactation. In support of this, it has been observed that SGA infants have a reduced kidney length at birth which is normalised by the 3rd to the 24th month of life (Giapros et al. 2006). This suggests that the kidney undergoes catch up growth following growth

restriction at birth, and highlights that catch-up growth is likely responsible for the increase in kidney weight and area seen in offspring of obese dams in the current study.

A decrease in glomeruli density was also observed in offspring of obese dams. This was likely due to the increase in cortex area because there was no difference in total nephron number between the two offspring groups. The increase in body weight of offspring of obese dams could be seen from around PN14 and it is likely that kidney growth mirrored overall growth. In the mouse, it has been reported that nephrogenesis is completed from a few days after birth (Costantini 2010; Hokke, Arias, et al. 2016) to around 10-14 days after birth (Slattery et al. 2016). It is known that kidney size increases following nephrogenesis due to a combination of tubular hypertrophy, hyperplasia and interstitial expansion (Sadler 2011; Seely 2017). These changes are necessary for functional maturation of the kidney and to cope with the increasing metabolic demand of the growing infant. Therefore, it is likely that kidney catch-up growth stimulated tubule growth in the latter part of lactation after nephrogenesis completion, leads to the reduction in glomeruli density. This has potential implications for the offspring of obese dams in this study. Tubule maturation involves the development of ion transporters such that a change in environment affecting tubule growth could permanently program ion homeostasis. Accordingly, increased tubular ion transport or increased ion channel expression has been seen in growth restricted rats exposed to a maternal low protein diet (Bertram et al. 2001; Manning et al. 2002). Exposure to maternal diabetes has also been documented to increase ion channels in rat offspring (Nehiri et al. 2008). In all of these studies, the offspring developed hypertension suggesting that tubule programming leading to inadequate salt excretion might be one cause of the high blood pressure commonly seen in offspring exposed to adverse early life environments including offspring in the current model (chapter 5). The cortex overgrowth/decrease in glomeruli density observed in offspring of obese dams in the current study could therefore be an indication that tubule morphology or function may be altered. It's important that this possibility is investigated in the future since adverse programming of the kidney tubules could increase the risk for CVD and renal disease in offspring of obese dams.

Most importantly, 3 week offspring of obese dams showed glomerular hypertrophy when compared to offspring of control dams. Glomerular hypertrophy is a hallmark of increased intra-glomerular pressure leading to hyperfiltration (Cullen-McEwen et al. 2003; Rocco et al. 2008; Helal et al. 2012). Individual glomerular hyperfiltration is often observed in models of nephron deficit (see Table in Chapter 1). In this setting, hyperfiltration is seen as compensatory in order to maintain the overall filtration demands of the whole kidney. Although offspring of obese dams in the current study had the same number of total nephrons as offspring of control dams by weaning, they were also heavier by this time. Glomerular tuft volume has been shown to increase exponentially with body weight in rats

due to increased filtration demand (D'Agati et al. 2016). This suggests that glomerular hypertrophy could have developed in offspring of obese dams due to the increased functional demand placed on each glomerulus by an increase in body weight.

Another potential factor leading to the glomerular hypertrophy seen in offspring of obese dams is insulin. Offspring of obese dams showed hyperinsulinaemia and it is generally accepted that insulin promotes glomerular filtration through NO dependent vasodilation (Hale & Coward 2013; D'Agati et al. 2016). Furthermore, insulin could promote glomerular hypertrophy directly by the synthesis of growth factors such as Insulin-like growth factors (IGF) 1 and 2 (Kambham et al. 2001). Therefore the hyperinsulinaemia in offspring of obese dams is consistent with the increase in glomerular size observed.

Hypertension is another factor known to increase intra-glomerular pressure leading to glomerular hypertrophy and hyperfiltration. Blood pressure wasn't measured in the offspring in this study due to the difficulties associated with the technique in young mice (8 weeks being the earliest it is technically feasible to measure mouse blood pressure). However, we and others have shown that young adult offspring of obese dams have increased blood pressure (Samuelsson et al. 2009) (see Chapter 5). Increased blood pressure has been observed together with glomerular hypertrophy in rat offspring following catch-up growth (Boubred et al. 2009), and several studies have shown hypertension and glomerular hypertrophy in offspring exposed to maternal diabetes (Rocha et al. 2005; Magaton et al. 2007; Rocco et al. 2008). These observations emphasise that increased blood pressure could be a contributing factor to the glomerular hypertrophy seen in offspring of obese dams at weaning in the current study. Furthermore, high blood pressure would be consistent with the hyperinsulinaemia seen in offspring of obese dams in this study since insulin promotes sympathetic nerve activity and sodium re-uptake in the renal tubules (D'Agati et al. 2016). Leptin also promotes increases in blood pressure through stimulation of the sympathetic nervous system in the hypothalamus (Simonds et al. 2014), which causes peripheral vasoconstriction and increases in renal sympathetic nerve activity which promotes ion re-uptake (D'Agati et al. 2016). Although leptin wasn't measured in offspring in the current study, offspring of obese dams showed increases in fat mass suggesting that leptin would also be increased.

Importantly, in countless models of exposure to adverse early life environments, glomerular hypertrophy has been observed in offspring together with either markers of impaired renal function or renal disease (see Table 1.2 in Chapter 1). In models of maternal diabetes, offspring glomerular hypertrophy has been seen together with decreases in GFR and renal plasma flow in the absence of any nephron deficit (Rocha et al. 2005; Magaton et al. 2007). Additionally, glomerular hypertrophy has been demonstrated to be a predictor of later life glomerulosclerosis (Fogo et al. 1990; Muda et al.

2017). It has been proposed that this damage occurs because hypertrophy increases capillary wall tension putting strain on the glomerulus over time (Grady & Novick 1996). This evidence suggests that by weaning, offspring of obese dams already show signs that they may be at risk of poor renal health in later life.

4.5.4 Limitations and future directions

One important limitation of this study was the measurement of total nephron number. Following total nephron counting, it was observed that the total numbers fell short of those published for wildtype mice (Cullen-McEwen et al. 2001; Cullen-McEwen et al. 2003). We repeated exhaustive sectioning and the counting protocol in a new 3 week control mouse kidney and found that the total nephron number was almost double that observed previously (Figure 4.5.A). We obtained many more kidney sections, resulting in more section pairs for counting, from the new control mouse despite no differences in kidney weight. It became apparent that the core histologist who performed the original sectioning had trimmed excessively the kidney. Since precise exhaustive sectioning is integral to the accurate estimation of total nephron number, we cannot rule out the possibility that offspring exposed to maternal obesity show a programmed nephron deficit. Indeed, these offspring show growth restriction at birth, glomerular hypertrophy and hypertension (see Chapter 5), and all these factors have been previously associated with a reduction in nephron number. Therefore going forward, it would be valuable to re-count nephrons in offspring exposed to maternal obesity. However, it's important to note that the glomerular morphology and density were consistent throughout kidney sections from each individual animal, suggesting that the total nephron number counted in this study was reflective of the true value. Secondly, the total medulla and cortex volume from which total nephron number was calculated was significantly increased in offspring from obese dams (Figure 4.5.B), consistent with the increased cortex and medulla areas and increased total kidney weight. Since glomeruli density was also reduced in offspring of obese dams, it makes sense that total nephron number would be unchanged. In support of this, in 8 week old animals where kidney weights and medulla and cortex areas were unchanged between the two offspring groups, glomerular density was also unchanged (see chapter 5). These observations increase the likelihood that the total nephron number presented in 3 week animals in the current chapter is reflective (at least for the purpose of comparing control and obesity exposed offspring) of the true total nephron number.

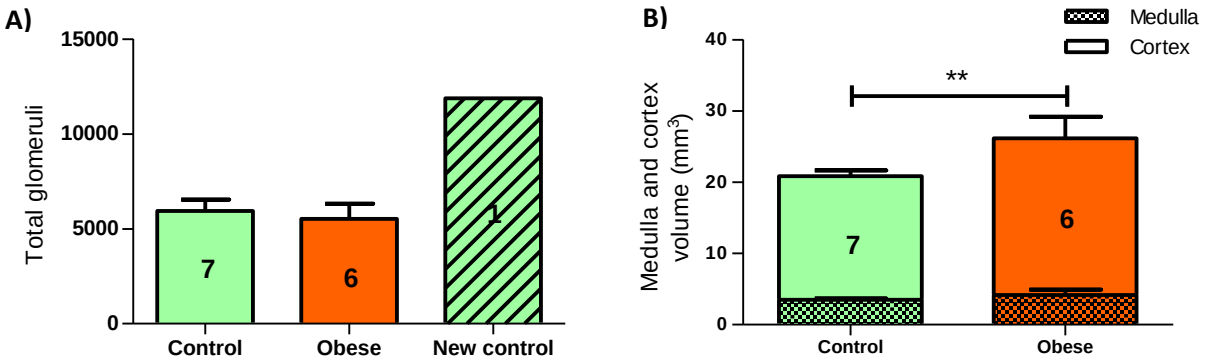


Figure 4.5. **Troubleshooting of total glomeruli number in 3 week old males. A)** Total glomeruli number in the old cohort of control and obese offspring and in one new control sample. **B)** The medulla and cortex volumes from which total glomeruli number was calculated in control and obese offspring. N numbers are presented within the graphs. Students T test; $p<0.001$.

Since there is evidence that the kidneys of offspring of obese dams under-went catch up growth with particular growth of the cortex, in the future it would be interesting to examine whether the tubules of offspring of obese dams have any programmed alterations in functions such as sodium handling. Alterations in ion handling have been observed numerous times in other models of adverse early life environments, including models resulting in offspring growth restriction at birth (see Table 2.1, Chapter 1). Investigating tubular function might help explain the increased blood pressure observed in offspring exposed to maternal obesity in this model.

Finally, in the future using a miniature telemetry system to measure blood pressure in 3 week mice (Zayachkivsky et al. 2015) might help to explain the glomerular hypertrophy observed in offspring of obese dams at this age, as well as the renal damage observed in these animals in later life (discussed in Chapter 6).

4.5.5 Conclusions

The results presented in this study demonstrate that exposure to maternal diet-induced obesity throughout gestation and lactation is sufficient to increase body weight, fat mass and serological markers of adverse metabolism in male offspring at weaning. Importantly, maternal obesity led to an alteration in renal morphology in exposed offspring including decreased glomeruli density and increased glomerular size. Going forward, it will be important to determine whether the total number

of nephrons is also altered in offspring of obese dams and whether any early life changes in renal morphology may influence the risk of hypertension and kidney dysfunction/disease in later life.

4.5.6 Summary

- Offspring exposed to maternal obesity showed growth restriction at birth but underwent catch-up growth and were heavier than control offspring by weaning.
- Offspring of obese dams showed increased fat pad mass as well as increased fasting serum cholesterol, triglyceride, insulin and glucose levels at weaning.
- Offspring of obese dams showed altered renal morphology at weaning including decreased glomeruli density and increased glomerular areas.
- Programmed renal morphological changes in early life could promote hypertension and renal dysfunction/disease in later life.

Chapter 5. Maternal obesity leads to increased offspring blood pressure in young adulthood

5.1 Introduction

Maternal obesity is now an established risk factor for development of metabolic syndrome in adulthood in exposed individuals. Although CKD has been increasing concurrently with obesity rates, the contribution of maternal obesity to kidney disease in adulthood has not been investigated in epidemiological studies and there are only limited studies in animal models.

5.1.1 Animal models show that early life over-nutrition can impact on heart and renal health in young adulthood

5.1.1.1 Animal models of maternal diabetes

Animal models of maternal diabetes demonstrate that offspring kidney and cardiovascular health can be impaired in young adulthood. Rats exposed to maternal diabetes (induced by a single dose of STZ prior to mating) demonstrated a reduced GFR and renal plasma flow from around 8-12 weeks of age (Rocha et al. 2005; Magaton et al. 2007; Rocco et al. 2008). Increased renal vascular resistance and immune cell infiltration has also been shown in 12 week old rat offspring exposed to maternal diabetes (Rocco et al. 2008). These studies show that following an early life insult such as maternal diabetes, the offspring kidney may struggle to function optimally from a relatively early age. It is known that poor renal health promotes hypertension and vice versa. Accordingly, rodent offspring exposed to maternal diabetes induced by a single dose of STZ have also been observed to be hypertensive by 8 weeks of age (Rocha et al. 2005; Magaton et al. 2007; Y. W. Chen et al. 2010). These studies highlight that early life exposure to maternal diabetes can have a drastic impact on both renal and cardiovascular health during young adult life.

5.1.1.2 Animal models of maternal high fat diet

Studies utilising maternal high fat diets during gestation and lactation also suggest that cardiovascular health could be influenced in exposed offspring early on in adult life. In rabbits, 4 month old offspring exposed to a maternal high fat diet demonstrated an increase in mean arterial pressure, heart rate and renal sympathetic nerve activity (Prior et al. 2014). As an increase in blood pressure is an established cause of renal damage, renal heath could also be affected at this time in offspring exposed to maternal high fat feeding. Another study in mice also found that offspring exposed to a maternal

high fat diet showed renal fibrosis, inflammation and oxidative stress by 32 weeks of age (Glastras, Tsang, et al. 2016). Although these offspring were older, the damage found was already established, raising the question of whether blood pressure, renal morphological changes and damage could have occurred earlier in adult life.

5.1.1.3 Animal models of maternal obesogenic diet

Similar to findings with models of maternal diabetes and high fat diets, models using maternal obesogenic diets during pregnancy and lactation support the notion that heart and kidney health can be impaired in young adult offspring. Our laboratory has shown that male mice develop indices of cardiac hypertrophy including increased left ventricular wall volume and increased cardiomyocyte cell area by 3 weeks of age (Blackmore et al. 2015). Accordingly, these offspring showed systolic and diastolic dysfunction associated with sympathetic dominance by 12 weeks of age (Blackmore et al. 2015). Studies in rats have also demonstrated that renal health is impaired by maternal obesity in young adult offspring. As discussed in chapter 4, section 4.1.2.4, a maternal high fat and fructose diet leads to increased urinary albumin excretion, glomerulosclerosis and tubulointerstitial fibrosis in male rats, and a decrease in GFR in female rats by 17 weeks of age (Jackson et al. 2012; Flynn et al. 2013). By 17 weeks of age, rats are considered young adults. This suggests that renal damage could be detected in mice exposed to a maternal obesogenic diet by the equivalent age of 8 weeks.

5.1.2 Altered renal morphology in early life promotes poor renal health in adulthood

The data presented in chapter 4 demonstrate that renal morphology is altered in 3 week offspring exposed to maternal obesity. Data from both epidemiological and experimental studies suggest that early life changes in renal morphology could be a factor promoting renal dysfunction in adulthood.

5.1.2.1 Epidemiological studies

Epidemiological studies have highlighted the relationship between altered renal morphology in early life and hypertension and renal dysfunction in later life. As discussed in Chapter 1, section 1.4.1, middle aged individuals with hypertension showed a decrease in glomerular number and glomerular hypertrophy without an increase in obsolescent glomeruli, suggesting that these individuals had a nephron deficit from birth (Keller et al. 2003). Hypertensive individuals from an Aboriginal population also had fewer, hypertrophied glomeruli (Hoy et al. 2006). It is unclear from these studies whether altered renal morphology from birth caused the high blood pressure observed in adulthood or if it arises through separate mechanisms. However, these studies do emphasise that blood pressure should be assessed in cases where renal morphology is found to be altered. Another study

demonstrated using ultrasound, that kidney volume was reduced in LBW and SGA children and that this was associated with impaired renal function and increased salt sensitivity (Simonetti et al. 2008). Finally, individuals with unilateral renal agenesis and a normal contralateral kidney had a substantial risk of proteinuria, hypertension and decreased renal function (Ritz et al. 2011). These studies highlight a strong relationship between altered renal morphology and renal and cardiovascular function in adulthood.

5.1.2.2 Animal studies

Animal models have thus far mirrored epidemiological findings; countless studies of both maternal under and over-nutrition demonstrate an association between altered renal morphology and hypertension and renal dysfunction in exposed offspring. As discussed in Chapter 1, section 1.5.1 and summarised in Table 1.2, offspring exposed to maternal calorie restriction (Regina et al. 2001; Almeida & Mandarim-De-Lacerda 2005) or a low protein diet (Woods et al. 2001) are growth restricted at birth, have smaller kidneys, nephron deficit and glomerular hypertrophy. Importantly, these offspring develop a decrease in GFR (Regina et al. 2001; Woods et al. 2001; Almeida & Mandarim-De-Lacerda 2005) and an increase in albuminuria (Almeida & Mandarim-De-Lacerda 2005), indicative of impaired renal function. Offspring exposed to maternal calorie restriction also showed glomerulosclerosis (Regina et al. 2001). Likewise, sheep programmed to be hypertensive by dexamethasone exposure during gestation showed nephron deficit and glomerular hypertrophy. Accordingly, these animals had an accumulation of collagens type I and III within the interstitium and surrounding the vessels in the kidney cortex (Wintour et al. 2003), suggestive of progressive damage. A model of maternal diabetes has also highlighted a link between offspring nephron deficit and renal dysfunction. Rat offspring exposed to maternal diabetes induced by STZ at the beginning of gestation had fewer glomeruli and developed hypertension, proteinuria and a reduction in GFR (Nehiri et al. 2008). Importantly, a couple of studies have also shown that rats exposed to maternal diabetes display glomerular hypertrophy in the absence of nephron deficit, and these offspring also demonstrate a reduction in GFR and hypertension (Rocha et al. 2005; Magaton et al. 2007), highlighting that glomerular hypertrophy is an independent factor associated with renal and cardiovascular dysfunction. Together these studies in animals highlight that changes in renal morphology are predictive of renal and cardiovascular dysfunction in various models of adverse early life environments.

5.1.3 The value of studying the impact of maternal obesity on offspring renal health post-puberty

It is clear from the observations discussed above, that exposure to conditions of maternal over-nutrition such as diabetes or obesity can impair renal and cardiovascular health in rodent offspring in early adulthood. It is also evident that changes in renal morphology are strongly associated with renal and cardiovascular dysfunction. This raises the possibility that offspring exposed to maternal obesity in the current study may also show poor renal and cardiovascular health by young adulthood, especially since the data presented in chapter 4 demonstrate that these offspring are growth restricted at birth and show changes in renal morphology at 3 weeks of age. The aim of this chapter was therefore to assess blood pressure and markers of renal function and health in 8 week old offspring exposed to maternal obesity throughout gestation and lactation. As our model of maternal obesity is similar to maternal obesity in humans, findings may ultimately support the prevention of chronic kidney diseases in the future.

5.2 Aims

The aims of this chapter were:

4. To characterise the phenotype of offspring, exposed to either an obesogenic environment or control environment throughout gestation and lactation, from weaning until 8 weeks of age.
5. To determine the metabolic profile of offspring at 8 weeks of age.
6. To determine blood pressure and heart rate in offspring at 8 weeks of age.
7. To assess markers of renal morphology, function and damage in the kidneys of 8 week offspring.

5.3 Methods

5.3.1 Offspring phenotype up to 8 weeks

5.3.1.1 Offspring weight and organ weights

Body weight, fat and lean mass were measured weekly from 4 to 8 weeks of age. Fat and lean mass were determined using TD-NMR (minispec TD-NMR, Bruker Optics, MA). Heart weights and kidney weights were collected from animals at post-mortem at 8 weeks of age following a 4 hour fast.

5.3.1.2 Serology

Blood was collected by cardiac puncture following a 4 hour fast. The serum was prepared and analysed as described in chapter 2, section 2.3. Tail blood glucose levels were also measured after a 4 hour fast as described in chapter 2, section 2.2.

5.3.2 Serum arginine vasopressin

Arginine vasopressin (AVP) is a peptide hormone produced in the hypothalamus and released in conditions of dehydration in order to promote renal water conservation, osmoregulation and cardiovascular homeostasis. AVP is derived from its precursor, preprovasopressin, along with peptides neurophysin II and copeptin. AVP and copeptin are released into the bloodstream in equal amounts. Whilst AVP has a short half-life and is difficult to measure, copeptin remains stable following blood collection for a few days (Khan et al. 2007). Copeptin can therefore be used as a marker of AVP levels in serum.

Serum copeptin levels were determined by ELISA (Cloud Clone Corp, Katy, USA). The assay sensitivity was >24.7pg/ml. All samples were assayed in duplicate. The intra coefficient of variance for the assay was 7.5%.

5.3.3 Serum corticosterone

Since results in 3 week animals (chapter 4) suggested that offspring might have altered corticosterone levels following stress, in the current study serum from fed and fasted 8 week old animals was used for corticosterone analysis to assess both baseline levels and levels in response to a stressor respectively. Serum was sent to the mouse biochemistry phenotyping facility (Institute of Metabolic Science) and corticosterone was analysed by enzyme immunoassay (Immunodiagnosticsystems, Tyne

& Wear, UK). The lower limit of detection was 17ng/ml. The intra-coefficient of variance for the assay was 5.8%.

5.3.4 Cardiovascular function

Systolic blood pressure and heart rate were measured in 8 week mice by tail-cuff plethmograph. A thorough description of this method can be found in chapter 2, section 2.4.

5.3.5 Renal morphology analysis

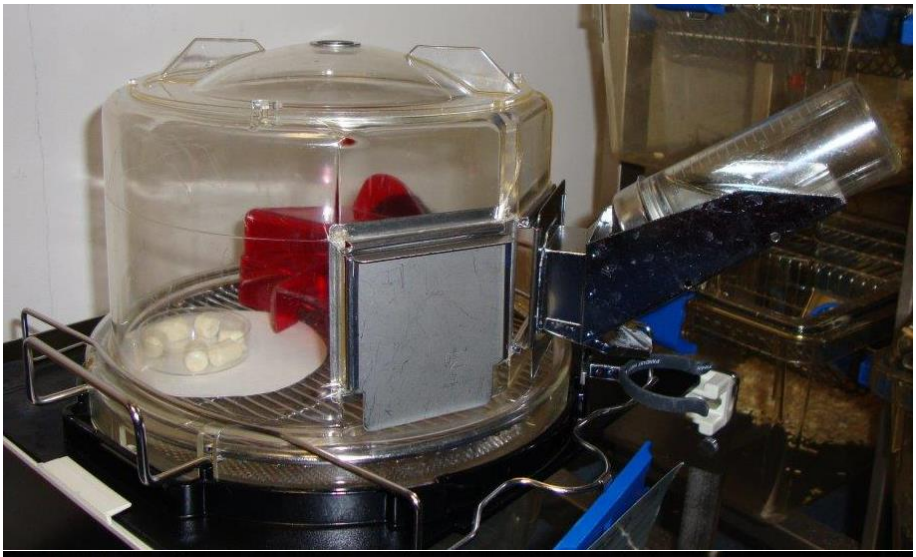
Processing, sectioning at 3µm, staining and imaging of tissue samples were all conducted as described in chapter 2, sections 2.5.1 - 2.5.4. Glomeruli areas and cortex/medulla areas were then calculated as described in chapter 2, sections 2.5.6 & 2.5.7 respectively. Glomeruli density was then calculated as described in Chapter 2, section 2.5.7.

5.3.6 Renal function

5.3.6.1 Urine collection

8 week old male mice were singly housed in metabolic cages to collect urine for 24 hours prior to post mortem (Figure 5.1). During this time males had free access to their assigned diet (chow or obesogenic) and water. After 24 hours, collected urine was filtered using a syringe and High Flow Syringe Filter (0.45 µm, Polyethersulfone, Sartorius Goettingen, Germany), before freezing at -20°C for later analysis.

A)



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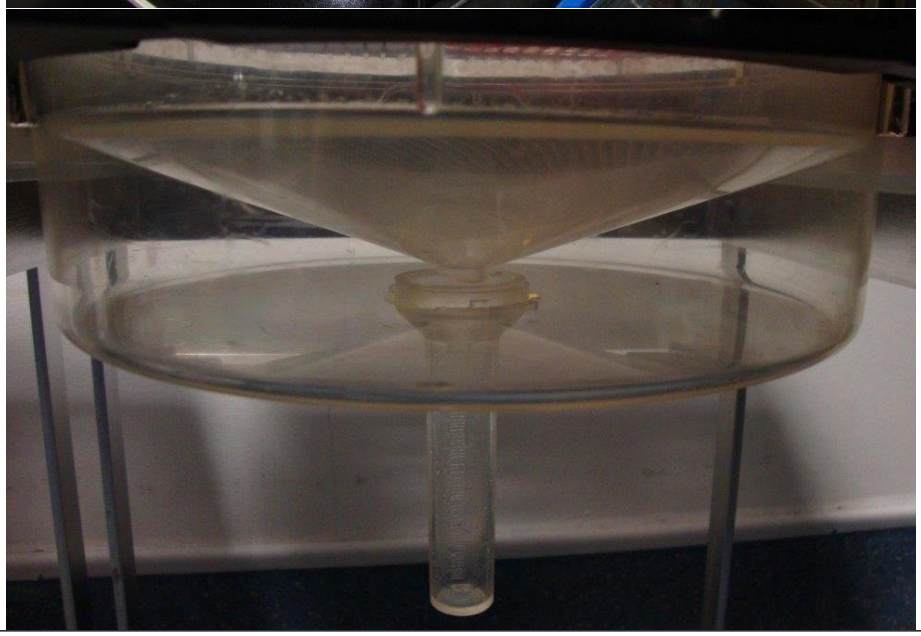

Figure 5.1. **Metabolic cage used for urine collection. A)** Top of the cage. 8 week male mice were placed in the cage with free access to food and water for 24 hours. The grate flooring of the cage allowed urine and faeces to pass through. A finer mesh layer beneath the grate trapped faeces. A separate compartment containing the water bottle ensured that no fresh water leaked into the cage and diluted the urine. **B)** The bottom of the cage. Urine passing through the floor of the cage was funneled and collected in a cylinder.

5.3.6.2 Urine and serum analysis

Following urine collection, mice were killed by rising CO_2 and the serum collected as described in chapter 2, section 2.3.1. Urine and serum was sent to the mouse biochemistry phenotyping facility (Institute of Metabolic Science) for analysis of urinary albumin, urinary and serum creatinine, and serum sodium, potassium and chloride. Creatinine levels were determined by enzymatic assay and measured using a Siemens Dimension EXL autoanalyser (Seiman's Healthcare Diagnotics, Sudbury, UK). The assay sensitivity was >3µmol/L. Urinary albumin levels were measured by ELISA (antibodies and standards provided by Bethyl laboratories, Montgomery, US). All samples were assayed in duplicate. The assay lower limit of detection was 7.8ng/ml. The intra coefficient of variance was 3.1%. Serum levels of sodium, chloride and potassium were determined by solid state electrode.

5.3.7 Renal fibrosis analysis

Processing, sectioning at 3µm, staining and imaging of tissue samples were all conducted as described in chapter 2, sections 2.5.1 - 2.5.4. Glomerulosclerosis and tubulointerstitial fibrosis were then determined as described in Chapter 2, section 2.5.5. Both glomerulosclerosis and tubulointerstitial fibrosis were expressed as the mean % stain on the images measured (see chapter 2, Figure 2.3).

5.3.8 Statistics

All statistical analyses were conducted using Prism 5.0 (GraphPad, CA, USA). All data were checked for normality by either a Shapiro-Wilk or a Kolmogorov–Smirnov (for smaller data sets) test. Normally distributed data were analysed by students T test and the mean and SEM are presented. Non-normal data including serum HDL, glomerulosclerosis and tubulointerstitial fibrosis were analysed by Mann Whitney tests and the median and IQR are presented. For all data sets $p<0.05$ was considered statistically significant. Sample sizes (n) are reported within each figure. For all data sets n represents the number of independent litters per experimental group. Importantly, only 1 male per litter at each time point was used to avoid litter bias.

5.4 Results

Maternal diet had no significant effect upon offspring body weight from 4 to 8 weeks of age (Figure 5.2.A). Lean mass was also unaffected by maternal diet from 4 to 8 weeks (Figure 5.2.B). However, exposure to maternal obesity significantly increased offspring fat mass from 4 to 6 weeks (Figure 5.2.C). At 7 weeks of age, offspring of obese dams remained fatter than offspring of control dams but this was only borderline significant (p=0.054; Figure 5.2.C). At 8 weeks, maternal obesity significantly increased offspring fat mass (Figure 5.2.C).

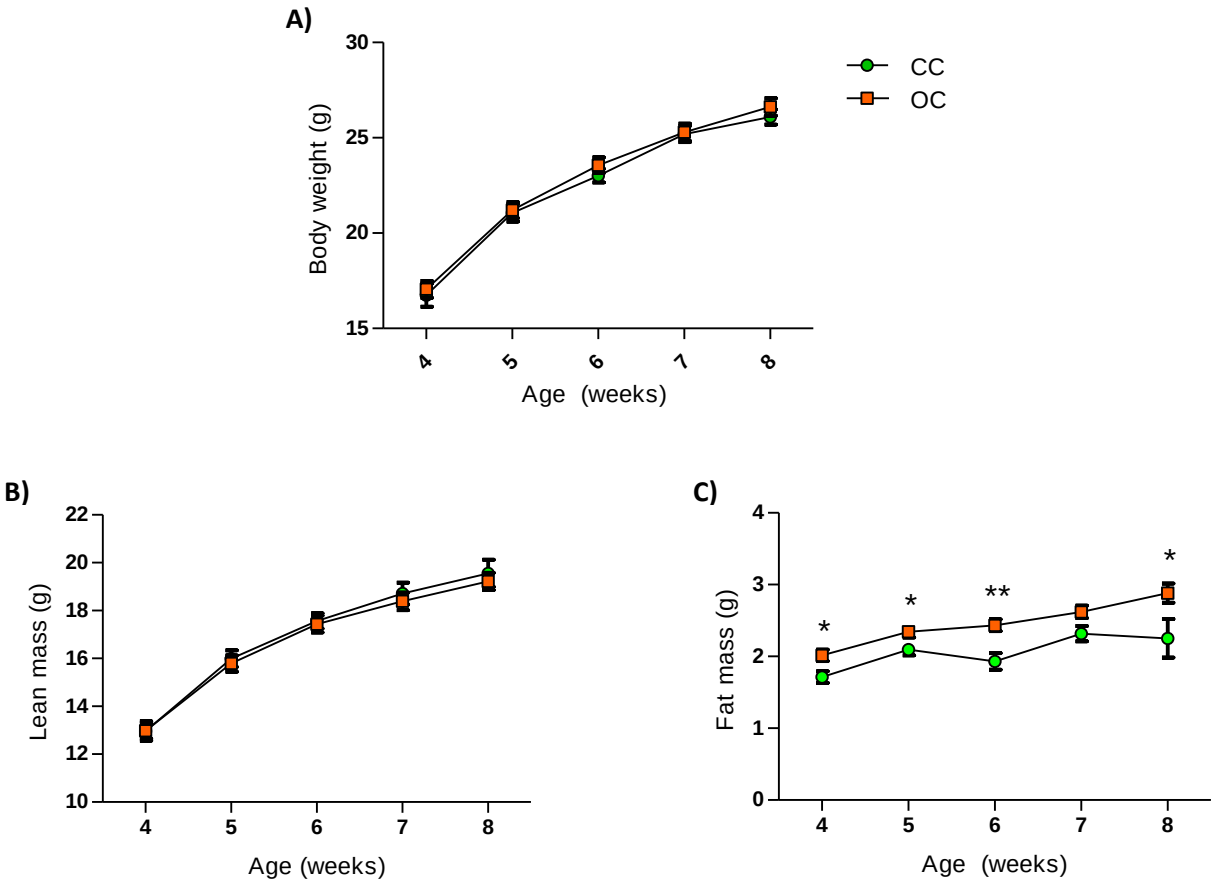

Figure 5.2. **Offspring body weight and composition from 4 to 8 weeks of age. A)** Offspring body weight. **B)** Offspring lean mass. **C)** Offspring fat mass. N = >7 CC, >18 OC. Students T test; *p<0.05, **p<0.001.

At 8 weeks, exposure to maternal obesity significantly increased offspring heart weight (Figure 5.3.A). This remained significant when heart weight was normalised to body weight (Figure 5.3.B). Maternal diet had no significant effect on offspring left kidney weight (Figure 5.3.C), right kidney weight (Figure 5.3.D) or total kidney weight (p=0.28; Figure 5.3.E). Total kidney weight normalised to body weight was also unaffected by maternal diet (p=0.38; Figure 5.3.F).

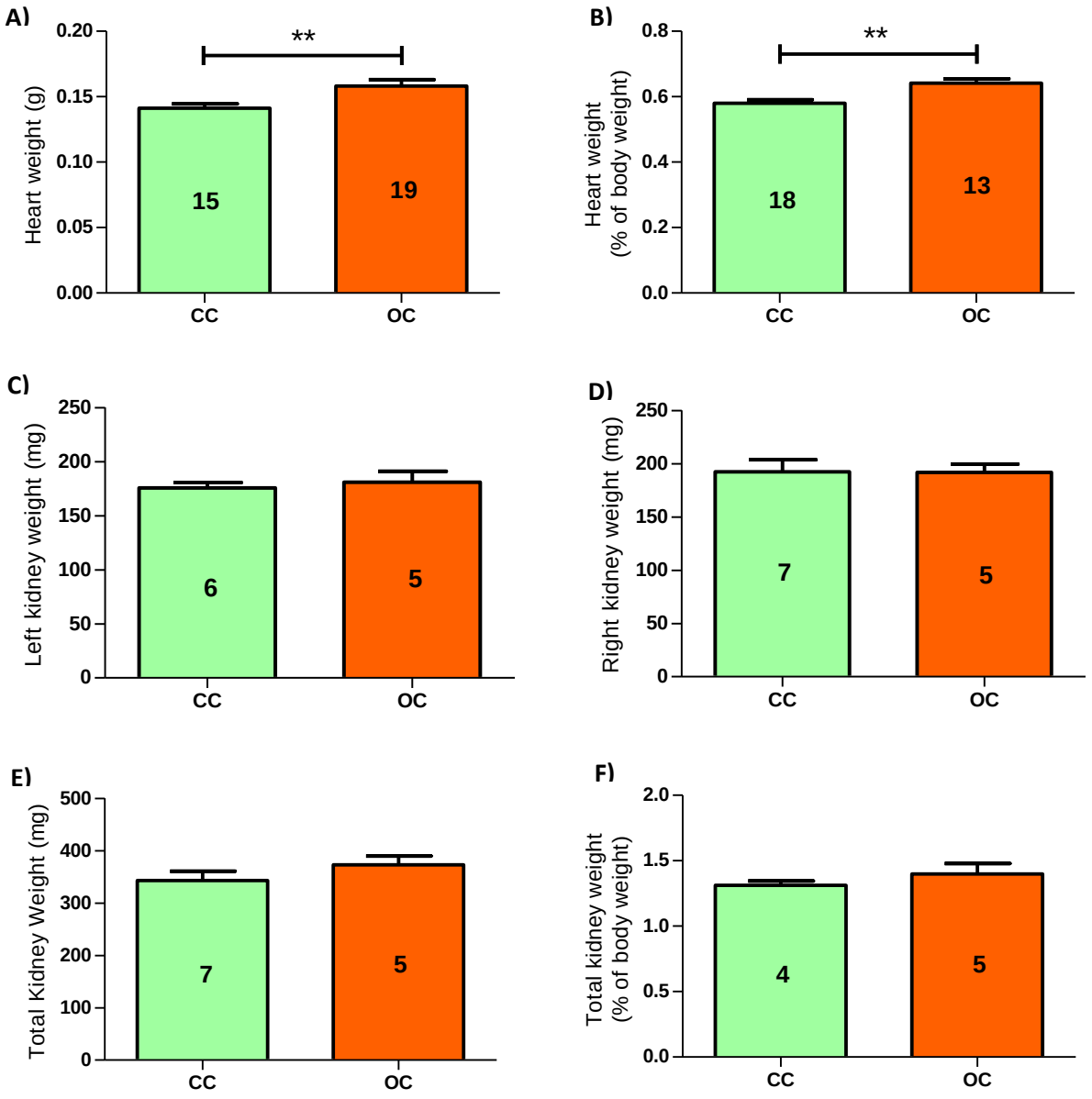

Figure 5.3. **Offspring organ weights at 8 weeks of age. A)** Heart weight. **B)** Heart weight normalised to body weight. **C)** Left kidney weight. **D)** Right kidney weight. **E)** Total kidney weight. **F)** Total kidney weight normalised to body weight. N numbers are presented within the graphs. Students T test; **p<0.001.

At 8 weeks of age, there were no significant differences in cholesterol, LDL or HDL in offspring exposed to maternal obesity when compared with control animals. Insulin and leptin levels were significantly increased by exposure to maternal obesity. Tail blood glucose levels were unaffected by maternal diet. These results are presented in Table 5.1.

Table 5.1. *Offspring serology measures at 8 weeks of age following a 4 hour fast.*

	CC (n=8-9)	**OC (n=7-8)**
Cholesterol (mmol/l)	3.27±0.1	3.29±0.09
LDL (mmol/l)	0.8±0.05	0.7±0.1
HDL (mmol/l)	1.88±0.21	2±0.35
Insulin (pmol/l)	119±6	168±14**
Leptin (ng/ml)	1.18±0.07	2.16±0.25**
Glucose (mmol/l)	11.4±0.5	10.4±0.7

N numbers are presented within the table. Mann-Whitney U test (HDL), Students T test (all other variables); ** $p<0.001$.

At 8 weeks of age, maternal diet had no significant effect on offspring serum copeptin levels although there was a trend toward significantly increased levels in offspring of obese dams (p = 0.067; Figure 5.4.A). Serum corticosterone levels were unaffected by maternal diet in either fed animals or fasted animals (Figure 5.4.B).

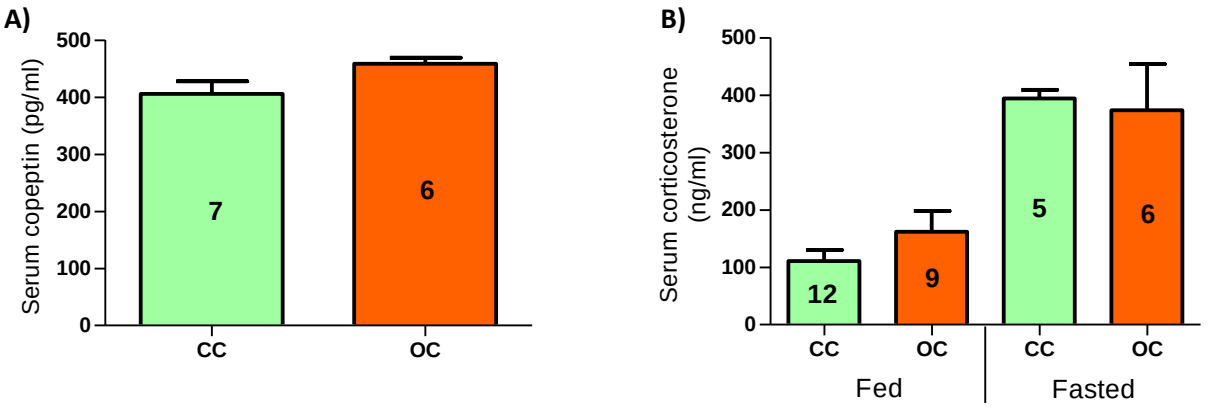

Figure 5.4. **Serum copeptin and corticosterone levels in offspring at 8 weeks of age. A)** Copeptin. **B)** Corticosterone. N numbers are presented within the graph. Students T test.

Exposure to maternal obesity during gestation and lactation significantly increased both offspring systolic blood pressure (Figure 5.5.A), and heart rate (Figure 5.5.B).

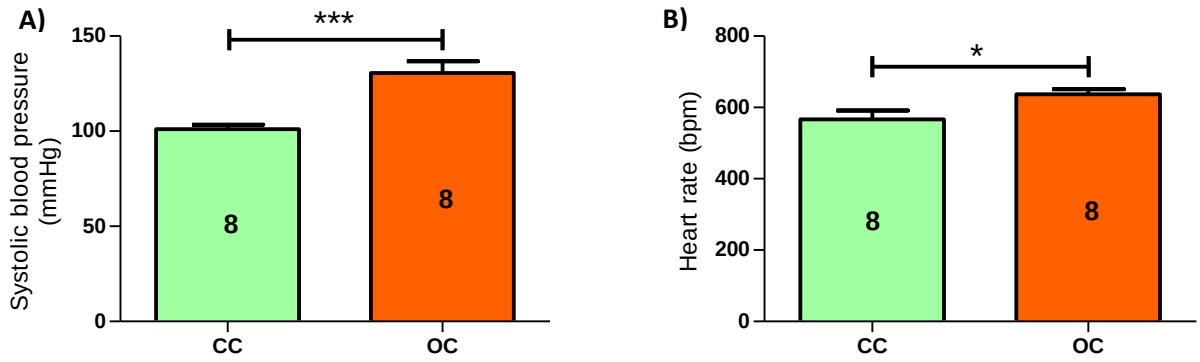

Figure 5.5. **Cardiovascular function in 8 week old offspring. A)** Systolic blood pressure. **B)** Heart rate. N numbers are presented within the graphs. Students T test; *p<0.05, *** p<0.0001.

At 8 weeks of age, maternal diet had no effect on offspring medulla and cortex area (Figure 5.6.A), or glomeruli density (Figure 5.6.B). However, exposure to maternal obesity significantly increased offspring glomeruli area (Figure 5.6.C).

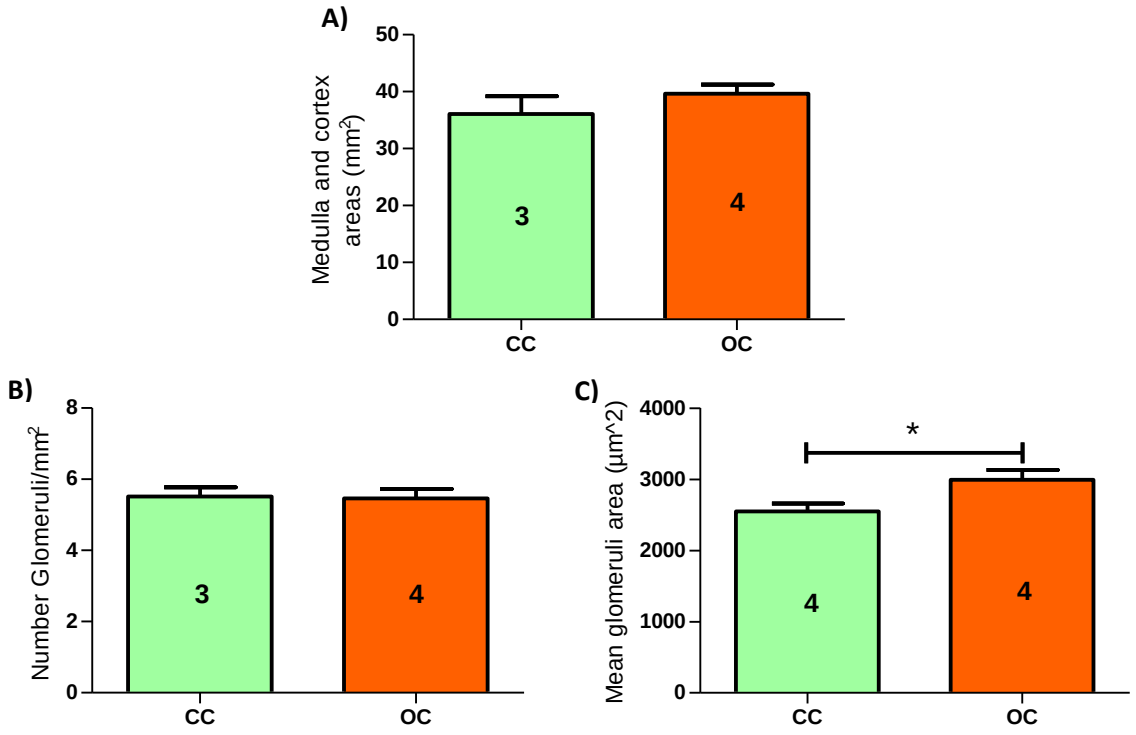

Figure 5.6. **Glomerular morphology in 8 week old offspring. A)** Glomeruli density. **B)** Mean glomeruli area. N numbers are presented within the graphs. Students T test (glomeruli area), Mann-Whitney U test (all other graphs); *p<0.05.

At 8 weeks of age, there was no significant effect of maternal obesity on offspring urinary creatinine levels (p=0.1801; Figure 5.7.A), serum creatinine levels (p= 0.52; Figure 5.7.B), or urinary albumin/ creatinine ratio (p=0.09; Figure 5.7.C). Serum sodium (Figure 5.7.D), chloride (Figure 5.7.E), and potassium (Figure 5.7.F) levels were also unaffected by maternal diet.

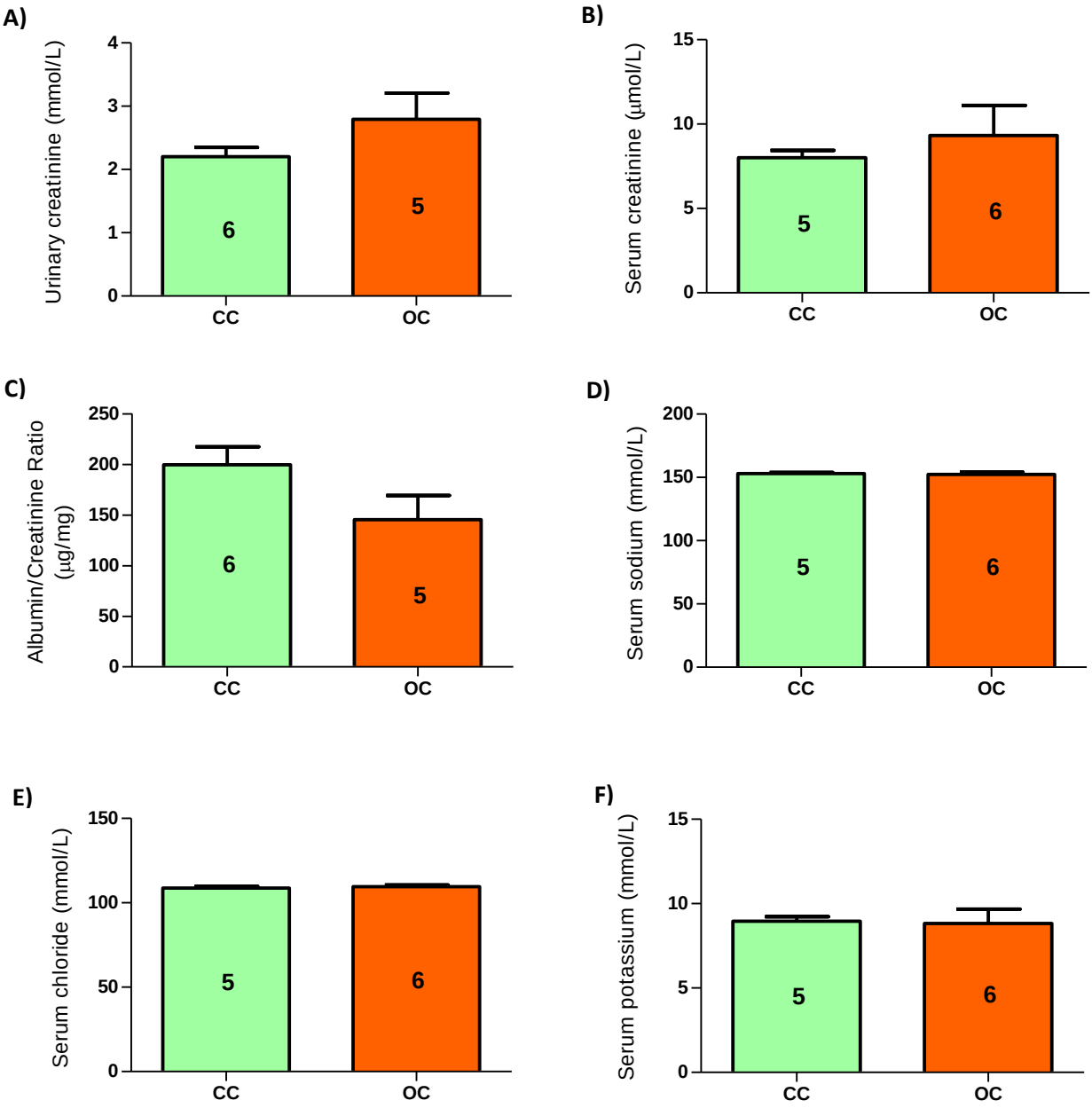

Figure 5.7. **Renal function in 8 week old offspring. A)** Urinary creatinine. **B)** Serum creatinine. **C)** Urinary albumin/creatinine ratio. **D)** Serum sodium. **E)** Serum chloride. **F)** Serum potassium. N numbers are presented within the graphs. Students T test.

Maternal obesity had no significant effect on offspring glomerulosclerosis as assessed by either masson's trichrome staining (Figure 5.7.A & B), or PAS staining (Figure 5.7.A & C). Tubulointerstitial fibrosis was also unaffected by exposure to maternal obesity in offspring as assessed by masson's trichrome staining (Figure 5.8.A & B) and PAS staining (Figure 5.8.A & C).

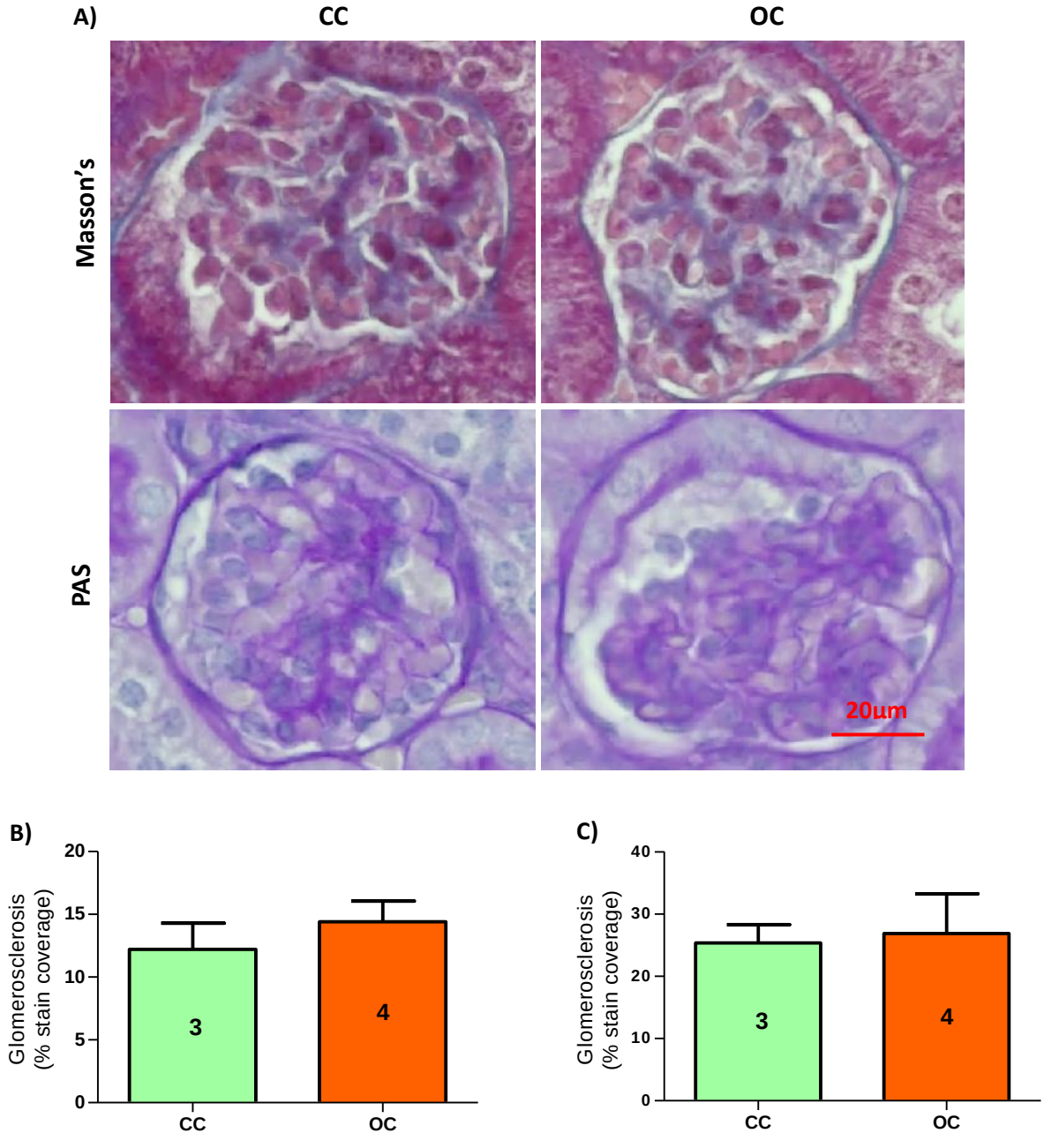

Figure 5.7. **Glomerulosclerosis in 8 week old offspring. A)** Masson's trichrome and PAS stained glomeruli from CC and OC males, blue and magenta stain respectively shows connective tissue. **B)** Mean % stain coverage within glomeruli following Masson's trichrome staining. **C)** Mean % stain coverage within glomeruli following PAS staining. N numbers are presented within the graphs. Mann-Whitney U test.

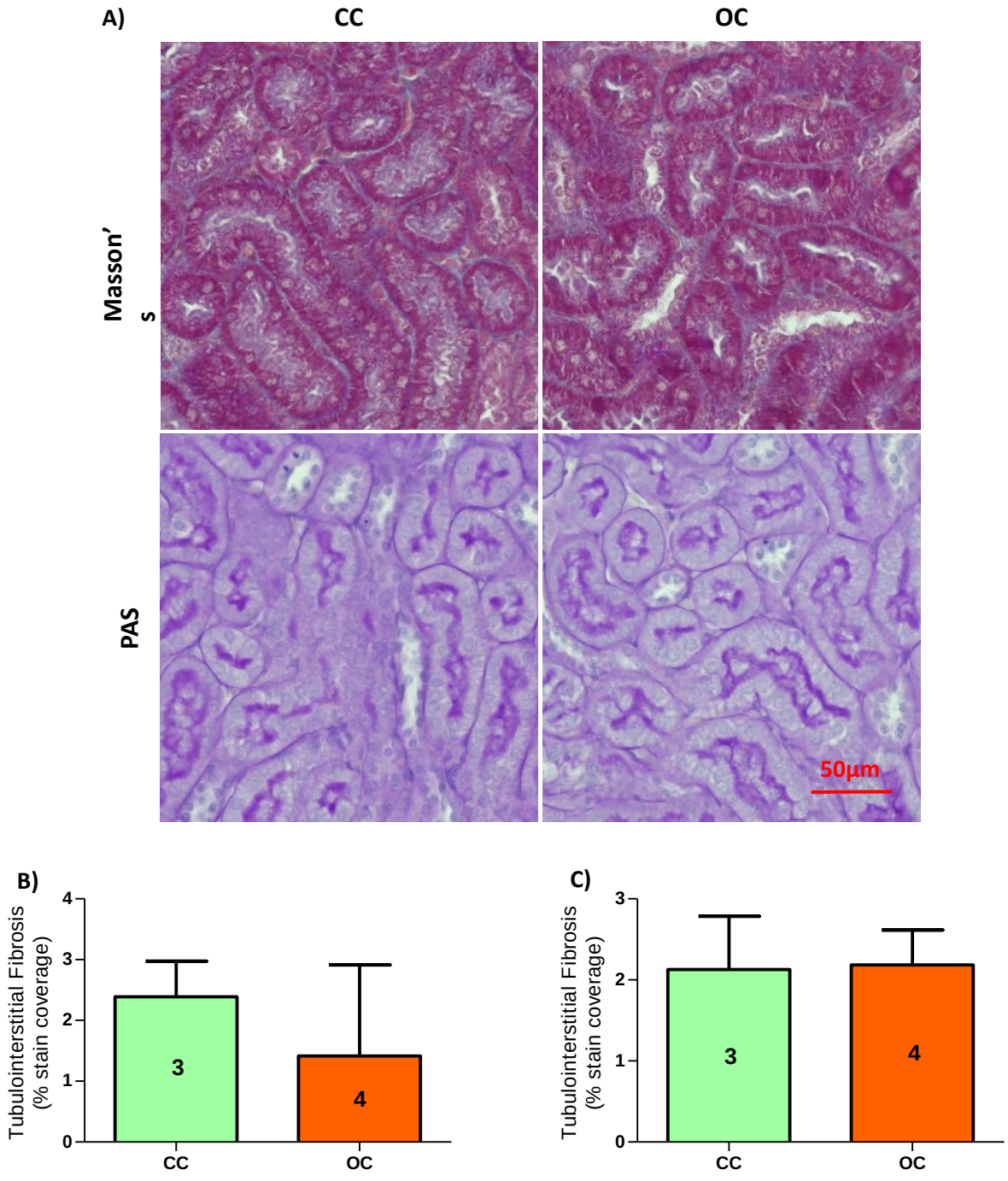

Figure 5.8. **Tubulointerstitial fibrosis in 8 week old offspring**. **A)** Masson's trichrome and PAS stained cortex from CC and OC offspring, blue and magenta stain show connective tissue respectively. **B)** Mean % stain coverage within the cortex (excluding glomeruli) following Masson's trichrome staining. **C)** Mean % stain coverage within the cortex (excluding glomeruli) following PAS staining. N numbers are presented within the graphs. Mann-Whitney U test.

5.5 Discussion

The aim of this chapter was to measure blood pressure, renal function and renal damage in young adult male mice exposed to maternal obesity throughout gestation and lactation and to compare these characteristics to offspring exposed to a maternal control diet.

5.5.1 Physical and metabolic phenotype

From 4 to 8 weeks of age, maternal diet had no effect on offspring weight. Despite this, offspring of obese dams showed a significant increase in fat mass without a change in lean mass. This is consistent with the increased fat pad mass demonstrated by these offspring at weaning (see chapter 4, section 4.4) and emphasises that maternal obesity imposes a sustained effect on offspring metabolism that lasts into adulthood. This finding is also in line with published results from another laboratory using an identical mouse model of maternal diet induced obesity, where exposed offspring showed increased inguinal fat mass at 12 weeks of age (Samuelsson et al. 2008).

Heart weight and relative heart weight were also increased in 8 week offspring exposed to maternal obesity in accordance with offspring of obese dams at weaning (chapter 4, section 4.4). This finding is consistent with data our laboratory has published previously; 8 week male mice exposed to maternal obesity had an increased heart weight to body weight ratio and increased cardiomyocyte cell area suggestive of cardiac hypertrophy (Blackmore et al. 2015). This is therefore a robust phenotype programmed by maternal diet-induced obesity.

Offspring exposed to maternal obesity also demonstrated elevated insulin and leptin levels concurrently with 3 week offspring (chapter 4, section 4.4). As discussed previously, increased leptin levels are reflective of the increased fat mass seen in these animals at this time. Increased insulin levels were seen in 8 week offspring of obese dams despite the fact that these offspring were weaned onto a healthy chow diet. As discussed in chapter 4, section 4.5.2, the increased insulin levels are consistent with the increased adiposity phenotype seen in these offspring. This finding also corroborates with another study demonstrating hyperinsulinemia together with increased fat mass in 12 week offspring exposed to maternal diet induced obesity (Samuelsson et al. 2008). These results emphasise that exposure to maternal obesity in early life is sufficient to alter the offspring metabolic health through to early adulthood independently of changes in body weight.

5.5.2 Cardiovascular phenotype

Offspring of obese dams showed a trend towards increased serum copeptin levels, although the magnitude of the effect was small. As discussed in section 5.3.2, serum copeptin levels are directly proportional to serum AVP levels. Unpublished data from our laboratory indicate that AVP and corticotropin releasing hormone mRNA levels are significantly increased within the paraventricular nucleus of female offspring exposed to maternal obesity. This suggests that AVP may be dysregulated in offspring of both sexes exposed to maternal obesity. AVP binds to three different receptors, vasopressin receptor 1A (V1AR), vasopressin receptor 1B (V1BR) and vasopressin receptor 2 (V2R). V1AR stimulation on vascular smooth muscle cells promotes peripheral vasoconstriction (Henderson & Byron 2007). In the anterior pituitary, V1BR action is involved in HPA axis stimulation and the stress response, leading to corticotropin release and cortisol production (Tanoue et al. 2004). V2R stimulation in the kidney promotes water reabsorption through aquaporin 2 in the collecting duct as well as increased sodium uptake through the $Na^+/K^+/2Cl$ cotransporter in the thick ascending limb (Tamma et al. 2015). The actions of AVP through all three receptors promote increases in blood pressure and blood volume. The importance of AVP on raising blood pressure in mice has been demonstrated using receptor knock out models (Koshimizu et al. 2006; Aoyagi et al. 2009).

Maternal diet had no significant effect on either fasted or fed serum corticosterone levels in 8 week old offspring. This is in contrast to findings in 3 week animals, where offspring exposed to maternal obesity showed decreased corticosterone levels relative to control animals following a fast (chapter 4). One possible explanation for this discrepancy is that 3 week old animals exposed to maternal obesity showed decreased corticosterone levels relative to control animals due to accelerated postnatal catch up growth and an associated early decline in the postnatal corticosterone surge. This is discussed in chapter 4, section 4.5.2. In support of this, fasting corticosterone levels decreased in control animals from 3 weeks to 8 weeks of age, whereas levels in obesity exposed animals stayed roughly the same. Alternatively, as discussed in chapter 4, section 4.5.2, an altered corticosterone level, as observed in 3 week old animals exposed to maternal obesity, can be indicative of stress. Therefore 3 week old animals exposed to maternal obesity may have been more stressed following a fast than 8 week old offspring. This could have occurred due the fact that 3 week old offspring were deprived from an obesogenic diet during fasting whereas 8 week old offspring were deprived from a chow diet.

Blood pressure was also elevated in offspring of obese dams. This was consistent with the trend towards increased serum copeptin levels in offspring exposed to maternal obesity. This is in accordance with data from Samuelsson and colleagues demonstrating that male mice exposed to the same model of maternal diet induced obesity had increased systolic, diastolic and mean arterial blood

pressure by 12 weeks of age (Samuelsson et al. 2008). Our laboratory has also showed, using isolated Langendorff perfusion, that 12 week mice exposed to maternal obesity had systolic and diastolic dysfunction which was associated with sympathetic dominance (Blackmore et al. 2015). The current data together with previously published findings emphasise that maternal obesity predisposes offspring for cardiovascular dysfunction in young adulthood. Since hypertension can cause stroke, heart failure and kidney failure and is the leading cause of mortality globally (Tu et al., 2008), these results indicate that exposure to maternal obesity may have severe implications for offspring health later on in life, even if offspring have a healthy post-maternal environment.

Heart rate was also increased in offspring exposed to maternal obesity. This is in contrast with previous findings in this model published by others in 12 week old offspring using telemetry methodology that demonstrated that heart rate was decreased (Samuelsson et al. 2008). Indeed, heart rate would be expected to decrease in response to increasing blood pressure in conditions of a normal baroreceptor response. An increase in both blood pressure and heart rate, as seen in offspring exposed to maternal obesity in the current study, could therefore indicate an impaired baroreceptor response. Alternatively, the increased blood pressure and heart rate could reflect increased offspring leptin levels. In diet-induced obesity, leptin has been demonstrated to directly increase both blood pressure and heart rate through central stimulation of the sympathetic nervous system (Simonds et al. 2014). Additionally, an alteration in selective central leptin sensitivity in offspring of obese dams could also promote increased blood pressure and heart rate. In support of this, 17 week rabbit offspring exposed to a maternal high fat diet had increased blood pressure and heart rate together with increased renal sympathetic nerve activity and an altered central response to leptin. Whereas anorexic effects were impaired in response to central leptin administration to high fat diet exposed offspring, cardiovascular sympathetic responses were enhanced (Prior et al. 2014). Additionally, in mice it has been demonstrated that hyperleptinaemia during lactation increased blood pressure in a similar manner to offspring exposed to maternal obesity (Samuelsson et al. 2016). These findings are consistent with the increased blood pressure, heart rate and leptin levels seen in offspring of obese dams in the current study.

Another possible explanation for an increase in both blood presure and heart rate is an altered stress response. Rat offspring exposed to a maternal high fat diet throughout gestation and lactation had a normal baseline blood pressure, but had an increased blood pressure and heart rate in response to restraint stress (Rudyk et al. 2011). Adrenergic receptor blockade normalised these increases, suggesting offspring had sympathetic over-activity in response to stressful stimuli. It's possible that offspring exposed to maternal obesity in the current study also showed an increased heart rate and blood pressure due to stress induced sympathetic stimulation. Blood pressure and heart rate were

measured by tail-cuff plethysmography which involves placing the mice in a restrainer. Mice were acclimatised to the restraint using standard methods before measurements were conducted. However, it has been suggested that blood pressure measurement by the tail-cuff method creates at least a mildly stressful condition (Wlodek et al. 2007). It's therefore possible that offspring of obese dams were more stressed in response to restraint compared with control offspring. Indeed an increase in blood pressure and heart rate due to stress would be consistent with the increase in AVP and corticotropin-releasing hormone (CRH) in the hypothalamus seen in offspring exposed to maternal obesity in our model (unpublished- see above), which is suggestive of altered HPA action. It would also correspond with several other studies in both humans and animals demonstrating that exposure to a maternal high fat diet increases the stress response in offspring (Sasaki et al. 2013; Sullivan et al. 2015; Grissom et al. 2017; Mina et al. 2017). The evidence discussed suggests that offspring of obese dams are more at risk of cardiovascular dysfunction possibly due to alterations in central leptin signalling, sympathetic over-activity and HPA axis alteration.

5.5.3 Renal phenotype

5.5.3.4 Renal morphology

Maternal diet had no effect on offspring cortex and medulla areas at 8 weeks of age. This is in contrast with findings at 3 weeks (chapter 4, section 4.4), where offspring of obese dams showed an increased in cortex and medulla areas compared to control offspring. The current data is consistent with the catch up kidney growth hypothesis described in chapter 4, section 4.5.3. From 3 to 8 weeks, medulla and cortex areas increased in both groups of offspring, however it appears that relatively more growth occurred in control offspring over this time period. This would suggest, as discussed in chapter 4, section 5.5.3, that tubule growth and development was accelerated in offspring of obese dams during lactation. Whereas control offspring showed a slower growth phenotype, catching up to offspring of obese dams by 8 weeks.

Consistently, there were no differences in glomeruli density between the two offspring groups at 8 weeks. This finding also supports the tubule overgrowth hypothesis discussed in chapter 4, section 4.5.3. At 3 weeks of age cortex and medulla area was increased in offspring of obese dams such that the number of glomeruli found per unit area was decreased. However, at 8 weeks there was no change in cortex and medulla area such that glomeruli density was also unchanged. The current data supports the data presented in chapter 4 and emphasises that offspring of obese dams showed an accelerated kidney as well as overall growth phenotype in early life. As discussed in chapter 4, section 4.5.3, this may have important implications for tubule function and ion homeostasis in offspring of obese dams.

Importantly, in agreement with findings at 3 weeks, offspring of obese dams showed glomerular hypertrophy as defined by an increased glomerular area, at 8 weeks compared to control offspring. This hypertrophy is consistent with increased blood pressure, and insulin levels also observed in offspring of obese dams at this age (see Chapter 4, section 4.5.3 for an in depth discussion). The results also mirror experimental studies which demonstrate glomerular hypertrophy in offspring exposed to a variety of maternal adverse conditions (see Table 1.2 in chapter 1). Critically, glomerular volume has been demonstrated to be an independent predictor of poor renal health in humans. Glomerular hypertrophy has been identified as an integral feature of hypertensive nephropathy and appears to precede glomerulosclerosis (Hughson et al. 2014). Glomerular hypertrophy also had an independent association with decreasing GFR in the Pima Indian population (Lemley 2003). This evidence suggests that offspring exposed to maternal obesity in the current study may be predisposed for renal disease.

5.5.3.5 Renal function.

Despite the increase in blood pressure and glomerular area in offspring of obese dams at 8 weeks of age, renal function appears to be uninfluenced by maternal diet at this time. This is suggested by the fact that albumin excretion, serum creatinine and ions were similar between the two offspring groups. High blood pressure can promote albuminuria by increasing intra-glomerular pressure which places shear stress on the podocytes, leading them to undergo hypertrophy and causing detachment. This undermines the integrity of the filtration barrier allowing proteins to leak through (D'Agati et al. 2016). The fact that offspring of obese dams showed normal urinary albumin levels in the face of increased blood pressure and glomerular hypertrophy (indicative of increased glomerular pressure), suggests that in young adulthood the glomerular filtration barrier was functioning well in offspring of obese dams.

Creatinine is a breakdown product of creatinine phosphate in muscle and is produced at a fairly constant rate. It is then freely filtered by glomeruli and is not reabsorbed in the renal tubules (Leelahavanichkul et al. 2014). As such, it is an established marker for GFR. The fact that serum creatinine levels were normal in offspring of obese dams therefore indicates that at 8 weeks, glomerular filtration was intact. These results are in contrast with data in Sprague Dawley rats demonstrating that male offspring exposed to a maternal high fat and fructose diet had increased albumin excretion and decreased GFR by 17 weeks of age (Jackson et al. 2012). It's possible that the current findings differ due to differences in methods. Serum creatinine is an indirect measure of GFR and can be influenced by non-renal factors (Leelahavanichkul et al. 2014), whereas GFR measured by urinary or plasma clearance of exogenous markers, as conducted by Jackson and colleagues, is known to be precise (Stevens & Levey 2009). Alternatively, species dissimilarity could account for differences

in results. Indeed it has been reported that Sprague Dawley rats are fairly susceptible to renal fibrosis and injury leading to albuminuria, whereas C57BL6 mice are fairly resistant to fibrosis (Yang et al. 2010).

Serum ion levels were also unaffected by maternal obesity in 8 week offspring demonstrating that renal water homeostasis was normal. Serum sodium levels can be altered by pathological changes in AVP levels (Kovesdy 2012), highlighting the possibility that increased AVP could have promoted alterations in water balance and sodium concentrations in offspring of obese dams. However in the setting of CKD, serum sodium alterations usually only occur in the very late stages of disease following significant decreases in GFR (Kovesdy 2012). Serum potassium and chloride levels are also commonly altered in the later stages of CKD and are predictive of mortality (Hsieh et al. 2011; Mandai et al. 2017). Since impairments in renal water and ion homeostasis appear to develop in advanced renal disease, it is unsurprising that offspring of obese dams showed normal serum ion levels at 8 weeks of age, especially since at this time GFR appeared normal. The results of the current study demonstrate that in young adulthood, offspring exposed to maternal diet induced obesity showed optimal renal function, despite displaying factors such as glomerular hypertrophy which increase the risk for renal dysfunction.

5.5.3.6 Renal fibrosis

Despite showing glomerular hypertrophy, offspring of obese dams showed no evidence of glomerular fibrosis at 8 weeks of age. Since it has been suggested that glomerular hypertrophy precedes fibrosis, this suggests that at 8 weeks, offspring have not been exposed to increased intra-glomerular pressure long enough for fibrosis to develop. In support of this, studies of maternal calorie restriction (Regina et al. 2001) and postnatal catch up growth (Boubred et al. 2009) have showed that offspring have glomerular hypertrophy in young adulthood but develop glomerulosclerosis in later life. These studies emphasise that offspring exposed to maternal obesity in the current study may be at risk of glomerulosclerosis with age.

Tubulointerstitial fibrosis was also unaffected by maternal diet in young adult offspring. This again is unsurprising given that there is evidence that tubulointerstitial fibrosis is a marker of established renal disease. Tubulointerstitial fibrosis is the final common pathway for all kidney diseases leading to chronic renal failure and its presence correlates with impaired excretory function (Zeisberg & Neilson 2010). Given that renal function was unaltered in offspring of obese dams at 8 weeks, it's unlikely that tubulointerstitial fibrosis would have developed by this time. Furthermore, a major risk factor for interstitial fibrosis is albuminuria (Birn & Christensen 2006; Lindenmeyer et al. 2008), which was absent in offspring of obese dams at this age. Notably, in mice there is evidence that exposure to

maternal diabetes leads to tubulointerstitial fibrosis in exposed offspring by 20 weeks of age (Aliou et al. 2016). This highlights that offspring exposed to maternal obesity in the current study may develop interstitial fibrosis in later life.

5.5.4 Limitations and future directions

The evidence provided demonstrated that offspring of obese dams developed elevated blood pressure and heart rate. This could result from a number of mechanisms including altered central leptin pathways, sympathetic over-stimulation and HPA over-sensitivity. One small limitation of this study was that the tail-cuff method of measuring blood pressure and heart rate may stress the mice such that blood pressure may not be measured at the true baseline. In the future it would therefore be valuable to employ telemetry as this would not only remove stress, but allow continuous monitoring which might highlight blood pressure and heart rate differences throughout the day and night as described by Samuelsson and colleagues (Samuelsson et al. 2008). Comparing results obtained from telemetry with tail-cuff methods may illuminate the contribution of stress to increased heart rate and blood pressure in offspring of obese dams.

It may also be useful to measure renal sympathetic nerve activity in order to assess the potential contribution of sympathetic over-stimulation to the increased heart rate and blood pressure seen in offspring of obese dams. Likewise, assessing the expression and/or activity of the components of central leptin pathways may reveal a role for altered leptin signalling in cardiovascular dysfunction.

Finally, a limitation of this study was that GFR was inferred from serum creatinine. Serum creatinine as an indicator of GFR has a few limitations, it may be influenced by non-renal factors including diet and muscle mass (Dharnidharka et al. 2002). In the future, it would be valuable to measure urinary clearance of an exogenous filtration marker such as inulin, which is considered the gold standard technique for measuring GFR (Stevens & Levey 2009). Although this technique is highly accurate, it is timely and costly, hence why endogenous markers of GFR such as creatinine are commonly utilised. Furthermore, it is unlikely that the limitations associated with creatinine are relevant to the current study since both experimental groups were the same age, had equivalent lean masses and had similar amounts of protein in their diets.

5.5.5 Conclusions

The results presented in this study demonstrate that exposure to maternal diet induced obesity throughout gestation and lactation is sufficient to increase fat mass and serological markers of altered metabolism in male offspring in young adulthood. Maternal obesity also increased heart to body weight ratio, increased blood pressure and promoted glomerular hypertrophy. These findings emphasise that the heart and kidneys of offspring exposed to maternal obesity are already under strain by young adulthood, and it is therefore important in future studies to assess cardiovascular and renal health in these animals as they age. These results imply that offspring exposed to maternal obesity are more at risk of cardiovascular and renal disease in later life.

5.5.6 Summary

- Offspring exposed to maternal obesity showed increased levels of fat mass, serum insulin and leptin by 8 weeks of age despite being weaned onto a healthy diet.
- Offspring of obese dams demonstrated increased blood pressure and heart rate.
- Offspring of obese dams showed glomerular hypertrophy.
- Hypertension and changes in glomerular morphology in young adulthood could lead to cardiovascular and renal disease in later life.

Chapter 6. Maternal obesity predisposes offspring for later life renal damage

6.1 Introduction

CKD is one of the fastest growing diseases globally and its rise is concurrent with increasing obesity prevalence. Obesity itself is an established risk factor for CKD, but the role of maternal obesity in promoting poor renal health in exposed offspring in later life has not been investigated in epidemiological studies and there are only limited studies in animal models.

6.1.1 Animal models of maternal over-nutrition show that the offspring kidney is damaged with ageing.

6.1.1.1 Maternal diabetes

Studies of maternal diabetes in rats have highlighted that the offspring kidney is susceptible to damage with ageing. Maternal diabetes induced in rats by STZ at the beginning of pregnancy led to an observable increase in offspring weight and hypertension after 6 months of age. Hypertension promotes poor renal health and vice versa. Accordingly, a decrease in glomerular filtration rate (GFR) was seen in diabetes exposed offspring between 6 – 18 months of age, and the most exaggerated increase in proteinuria in diabetes exposed offspring relative to control offspring was observed at 18 months of age (Nehiri et al. 2008). Likewise, rats exposed to maternal diabetes induced by a single dose of STZ 10 days prior to mating showed accelerated increases in body weight and blood pressure with age, with the largest differences between diabetes exposed and control offspring being observed at the end of the study (12 months of age). Notably, at 3 months of age diabetes exposed offspring had the same number of glomeruli as control offspring but by 12 months of age, diabetes exposed offspring had 20% fewer glomeruli. Ageing also decreased GFR and renal plasma flow, and impaired tubular acid excretion and sodium conservation in rats exposed to maternal diabetes (Rocha et al. 2005). These studies emphasise that ageing has a profound impact on offspring kidney health following exposure to maternal diabetes in early life. The ageing process may therefore expose or accentuate renal adversities that have been programmed in very early life.

6.1.1.2 Maternal high fat diet

Similarly to models of maternal diabetes, a study looking at the impact of a maternal high fat diet from 6 weeks prior to mating, throughout gestation and lactation demonstrated that offspring displayed renal damage in later life. As discussed in chapter 5, section 5.1.1.2, high fat diet exposed male mice

had increased tubulointerstitial injury and glomerulosclerosis by 32 weeks of age (Glastras, Tsang, et al. 2016). Renal inflammation (as assessed by Cluster of differentiation 68 (CD68) and EGF-like module-containing mucin-like hormone receptor-like 1 (F4/80) immunohistochemistry and DNA oxidation) was also increased in 32 week old offspring exposed to a maternal high fat diet. These results are indicative of substantial renal damage in offspring exposed to a maternal high fat diet by around middle age and are consistent with ageing being a stimulatory factor promoting renal disease in programmed offspring.

Studies of both maternal diabetes and high fat diets suggest that the offspring kidney can be adversely programmed and that some programmed detriments may be revealed with age. As discussed in chapter4, section 4.1.1.2, STZ is a poor model of the most common form of diabetes that currently complicates human pregnancy. Likewise, models of maternal high fat diet fail to adequately mimic the typical western diet (high in fats and simple sugars) that complicate human pregnancy. As discussed in chapter 3, our model of maternal diet-induced obesity utilising a high fat and sugar diet, is an ideal model of human obesity with GDM. To date, no animal model has investigated the impact of maternal diet-induced obesity on the offspring kidney with ageing.

6.1.2 Animal models of maternal over-nutrition show that offspring renal damage is exaggerated following a "second hit"

Many animal models have demonstrated that the offspring kidney is adversely programmed by a poor maternal environment. However these adversities may be revealed or exaggerated if the offspring are exposed to a secondary environmental insult such as a high fat or salt diet during later life.

6.1.2.1 Maternal diabetes

Animal models of maternal diabetes demonstrate that a secondary stressor in later life may exacerbate deficits in renal health programmed in early life. Rats exposed to maternal diabetes induced by STZ at the beginning of pregnancy showed a similar blood pressure as control animals at 3 months of age. However, after being challenged with a high salt diet, diabetes exposed animals had an increased blood pressure relative to animals exposed to a normal maternal environment (Nehiri et al. 2008). Diabetes exposed animals also showed a delayed increase in sodium excretion following a high salt diet. Accordingly, following salt loading, both β- and ϒ- Epithelial sodium channel subunits and Na^+/K^+ ATPase protein levels were significantly up-regulated in diabetes exposed offspring. These results suggest that in the event of an environmental challenge in adult life, the kidneys of offspring exposed to maternal diabetes have little capacity for adaptation and this results in a functional deficit

leading to salt sensitive hypertension. Likewise, another study demonstrated that rats exposed to maternal diabetes induced by a single dose of STZ at the beginning of pregnancy showed an accentuated increase in blood pressure following a high salt diet (Yan et al. 2014). N-acetyl-b-D-glucosaminidase (NAG) excretion was also highest in rats exposed both to maternal diabetes and a high salt diet suggesting that these animals showed the most extensive tubular damage. Together these studies demonstrate that maternal diabetes and a high salt diet have additive detrimental effects on offspring blood pressure, renal function and damage.

A model of maternal diabetes has also shown that a later life high fat diet can accentuate programmed renal adversities in offspring. Mice exposed to a single dose of STZ at E13 and fed a post-weaning high fat diet showed the most exaggerated increase in body weight of any experimental group throughout the study period (Aliou et al. 2016). These animals also showed the worst levels of glomerulosclerosis and tubulointerstitial fibrosis. Again this study emphasises that exposure to maternal diabetes in early life may predispose offspring for renal damage in later life, especially when these offspring are challenged with a secondary environmental insult.

6.1.2.2 Maternal high fat diet.

As discussed above, mice exposed to a maternal high fat diet had increased renal fibrosis, inflammation and oxidative stress compared with control offspring (Glastras, Tsang, et al. 2016). However, offspring exposed to a maternal high fat diet and also given a low dose of STZ for 5 consecutive days at 8 weeks of age, showed augmented protein levels of collagen type IV and fibronectin, renal inflammation and oxidative stress as compared with offspring exposed to a maternal high fat diet alone. Not only does this evidence highlight that a maternal high fat diet is sufficient to adversely program the offspring kidney, it also emphasises that environmental challenges experienced in adult life can exacerbate adversities laid down in early life.

6.1.2.3 Maternal obesogenic diet.

A couple of studies of maternal diet-induced obesity have provided additional evidence supporting the notion that the early life and later life environment can have additive effects on kidney health. As discussed in chapter 4, section 4.1.2.4, urinary albumin excretion was increased in male rats exposed to maternal obesity, together with glomerulosclerosis and tubulointerstitial fibrosis by 17 weeks of age (Jackson et al. 2012). In female rats of dams fed an obesogenic diet, GFR was decreased together with a decreased expression of nestin in the glomeruli suggestive of a reduction in podocyte number (Flynn et al. 2013). Importantly, in both of these studies a post-weaning obesogenic challenge amplified the adverse offspring renal phenotype programmed by maternal obesity. Males displayed a

more severe functional deficit together with increased fibrosis, whilst the females developed glomerulosclerosis, increased urinary albumin excretion and immune cell infiltration by 17 weeks of age. These studies provide further evidence that an adverse maternal environment primes the kidney for further damage when an insult is experienced in young post-weaning life, and highlights the need to study offspring exposed to such conditions with ageing.

6.1.3 The value of studying the effects of ageing/post-weaning obesogenic diet on the kidney in offspring exposed to maternal obesity.

It is clear from the evidence discussed above that the ageing process poses an additional risk for renal damage and dysfunction in offspring exposed to conditions of early life maternal over-nutrition including diabetes induced by STZ and high fat diet feeding. This raises the possibility that ageing might lead to renal damage in offspring exposed to maternal obesity in the current study, especially since offspring of obese dams showed increased blood pressure and glomerular hypertrophy at 8 weeks (see chapter 5), and hypertension and changes in glomerular morphology are often seen prior to overt renal damage. The evidence discussed above also highlights that secondary environmental insults following early life adverse environments can further damage the offspring kidney. This raises the possibility that a post-weaning obesogenic diet may also lead to kidney damage in offspring exposed to maternal obesity in the current study. The aim of this chapter was therefore to assess blood pressure and markers of renal function and damage in offspring exposed to maternal obesity in combination with ageing and/or a post-weaning obesogenic diet.

Studying the effect of maternal obesity in combination with post-weaning obesity on the offspring kidney is important given the nature of the current global obesity epidemic. There is now substantial evidence that exposure to a maternal western diet or obesity adversely programs the feeding habits of the offspring. Studies in both rodents (Parlee & MacDougald 2014) and non-human primates (Rivera et al. 2015) show that exposed offspring exhibit a tendency to over-eat as well as a preference for sugary and fatty foods. Children from obese families also show a preference for junk foods (Parlee & MacDougald 2014). These studies emphasise that, whether by early life programming or learned behaviour, offspring exposed to maternal obesity/western diet are more likely to eat more or seek an obesogenic diet themselves in later life. Therefore studying the effects of maternal diet-induced obesity in combination with a later life obesogenic diet on the offspring kidney is highly translatable and may support the prevention of renal diseases in future generations.

6.2 Aims

The aims of this chapter were:

- To characterise the phenotype of offspring exposed to maternal obesity and/or a post-weaning obesogenic diet up to 6 months of age.
- To assess blood pressure and Heart Rate (HR) in 6 month old offspring exposed to maternal obesity and/or a post-weaning obesogenic diet.
- To assess renal morphology in offspring exposed to maternal obesity and/or post-weaning obesity at 6 months of age.
- To measure renal function and indices of damage in offspring exposed to maternal obesity and/or post-weaning obesity at 6 months of age.

6.3 Methods

6.3.1 Offspring phenotype up to 6 months

6.3.1.1 Offspring weight and organ weights

Body weight was measured weekly from 4 to 12 weeks of age and monthly thereafter. Fat and lean mass were determined at the same time points using TD-NMR (minispec TD-NMR, Bruker Optics, MA). Organ weights were determined following a 4 hour fast and post-mortem.

6.3.1.2 Serology

Blood was collected by cardiac puncture following a 4 hour fast. The serum was prepared and analysed as described in chapter 2, sections 2.3.1 and 2.3.2. Tail blood glucose levels were also measured after a 4 hour fast as described in chapter 2, section 2.2.

6.3.2 Cardiovascular function

Systolic blood pressure and heart rate were measured using a tail-cuff plethysmography volume pressure recording system (BP-2000 Series II Blood Pressure Analysis System, Visitech systems). A detailed description of this method can be found in chapter 2, section 2.4.

6.3.3 Glomeruli and cortex morphology

Processing, sectioning at 3µm, staining with PAS and imaging were all conducted as described in chapter 2, sections 2.5.1 - 2.5.4. Glomeruli areas were then determined using image analysis software (Halo, Indica Labs, Corrales, USA) as described in chapter 2, section 2.5.6. Cortex and medulla areas and glomeruli density were measured as described in chapter 2, section 2.5.7.

6.3.4 Serum measures of renal function.

Cystatin C is a nonglycosylated cysteine protease produced at a constant rate by all nucleated cells (Dharnidharka et al. 2002). It is freely filtered by the renal glomeruli and catabolised by the renal tubules, making it an ideal serum marker of (GFR) (Coll et al. 2000). Following a 4 hour fast, serum was collected and stored at -80^{o}C as described in chapter 2, section 2.3.1. Serum cystatin C levels were

determined by ELISA (Abcam, Cambridge, UK). The assay sensitivity was >10pg/ml. All samples were assayed in duplicate. The intra-assay coefficient of variance was 7.4%.

Blood Urea Nitrogen (BUN) levels represent the balance between urea production and excretion. In the absence of factors that increase production (e.g. steroids and high protein diet), an increase in BUN reflects poor excretion. A decrease in urea excretion may indicate a decrease in GFR, or adverse haemodynamic, fluid volume balances and neurohormonal changes that promote urea reabsorption in the tubules (Cauthen et al. 2008). BUN levels were determined by colorimetric assay (BioAssay Systems, Hayward, USA). All samples were assayed in duplicate. The intra-assay coefficient of variance was 2.2%.

6.3.5 Intra-renal lipid content

Kidneys were collected from 6 month old animals after a 4 hour fast and stored at -80^{O}C prior to analysis. Renal tissue was powdered and the intra-renal lipid content was determined using the Folch method (Folch et al. 1957). Around 80mg of tissue was weighed to 1 decimal place into a fresh tube containing ceramic beads (lysing matrix D, 2ml tubes, MP Biomedicals, Santa Ana, USA). 1ml Folch mixture (666µl chloroform: 333µl methanol) was then added to the tissue and homogenised for 2 x 30 seconds using a ribolyser machine (Fast Prep-24, MP Biomedicals, Santa Ana, USA). 200µl water was then added to the tissue/Folch mixture and vortexed. The samples were placed on a shaker at 4^{o}C for 10 minutes before centrifuging for 10 minutes at 16100 x g to generate distinct organic and aqueous phases. 400µl was then removed using a pipette from the bottom lipid containing phase and placed into a clear glass tube that had been previously weighed in mg to 1 decimal place. The glass tubes were left on a Dri-Block heater (DB,2D, Bio-Techne, Abingdon, UK) at 37^{o}C overnight to allow the liquid to fully evaporate. The tubes were then weighed to give the combined lipid and glass tube weight. The lipid weight was calculated by subtracting the pre-determined glass tube weight from the combined lipid and glass tube weight. The % lipid in kidney tissue was then calculated as follows:

(Lipid weight x (666/400)/ tissue weight) x 100

666 is the amount of chloroform added to each sample and 400 denotes the amount of lipid containing solution removed at the end.

Samples were assayed over 2 days. A few random samples were repeated on both days to check the reproducibility of the method and to remove any variability generated by day. The assay inter-coefficient of variance for replicates was 8.6%.

6.3.6 Gene markers of renal damage

Kidneys were collected from 6 month old animals after a 4 hour fast and stored at -80^{O}C prior to analysis. RNA was extracted, cDNA generated and qPCR performed as described in chapter 2, section 2.6. Primers were designed as described in chapter 2, section 2.6.7. All primers were exon-exon spanning to avoid the amplification of genomic DNA. Primers were designed to detect the following genes: *Lpl* (lipoprotein lipase), *Fn1* (Fibronectin), *Mmp2* (Matrix metalloproteinase 2), *Casp12* (Caspase 12), *Ccl2* (Monocyte chemoattractant protein 1), *Kim1* (Kidney injury molecule 1). The expression level of these genes of interest were normalised to the geomean of the housekeeping genes (*Hprt, Gapdh* and *Sdha*) (the expression of which did not change between groups), as described in chapter 2, section. Table 6.1 shows the primers and their sequences used for 6 month renal gene expression detection.

Table 6.1. *Primer sequences for 6 month renal genes.*

Gene name	**Forward primer (5'→3')**	**Reverse primer (3'→5')**
Fn1	GGCCACCATTACTGGTCTGG	GGAAGGGTAACCAGTTGGGG
Mmp2	ACAAGTGGTCCGCGTAAAGT	GTAAACAAGGCTTCATGGGGG
Casp12	GGGTTTTTGATGACCTGGTGG	GAAAATTGTAAACTCAGCTGTTCCT
Ccl2	AGCTGTAGTTTTTGTCACCAAGC	GTGCTGAAGACCTTAGGGCA
Kim1	AGCTCAGGGTCTCCTTCACA	CACAGCGCCTGGGAGAAG
Hprt	ACAGGCCAGACTTTGTTGG	ACTTGCGCTCATCTTAGGT
Gapdh	CAACGGGAAGCCCATCAC	GCCTCACCCCATTTGATGTT
Sdha	TTACAAAGTGCGGGTCGATGA	TGTTCCCCAAACGGCTTCTT

6.3.7 Fibrosis quantification

6 month kidney tissue was fixed in formalin before being processed, sectioned at 3µm, stained with PAS and masson's trichrome, imaged and analysed as described in chapter 2, sections 2.5.1 – 2.5.5. Both glomerulosclerosis and tubulointerstitial fibrosis were expressed as the mean % stain on the images measured (see chapter 2, Figure 2.2).

6.3.8 Statistics

All statistical analyses were conducted using Prism 5.0 (GraphPad, CA, USA). All data were checked for normality by a Shapiro Wilk or Kolmogorov–Smirnov (for small data sets) tests. Statistical comparisons were performed using non-repeated measures 2-way ANOVA to assess the effects of both maternal and offspring diets, followed by Bonferroni post hoc testing. For all data sets, the mean and SEM are presented and $p<0.05$ was considered statistically significant. Sample sizes (n) are reported within each figure. For offspring data sets n represents the number of independent litters per experimental group. Importantly, only 1 male per litter at each time point was used to avoid litter bias.

6.4 Results

A post-weaning offspring obesogenic diet significantly increased offspring weight at all ages measured (Figure 6.1.A). Exposure to maternal obesity throughout gestation and lactation had no effect on body weight up to 12 weeks but significantly increased offspring weight from 16 weeks of age (Figure 6.1.B). TD-NMR analysis revealed that the increase in offspring weight due to maternal obesity was mainly due to an increase in offspring fat mass; offspring exposed to maternal obesity and a post-weaning control diet (OC) and offspring exposed to maternal obesity and a post-weaning obesogenic diet (OO) demonstrated increased fat deposition from around 12 weeks (Figure 6.1.B). An offspring obesogenic diet significantly increased fat mass from 6 weeks of age onwards (Figure 6.1. B). Lean mass was also significantly increased by an offspring obesogenic diet at all ages measured, whereas maternal obesity had no consistent effect on lean mass (Figure 6.1.C).

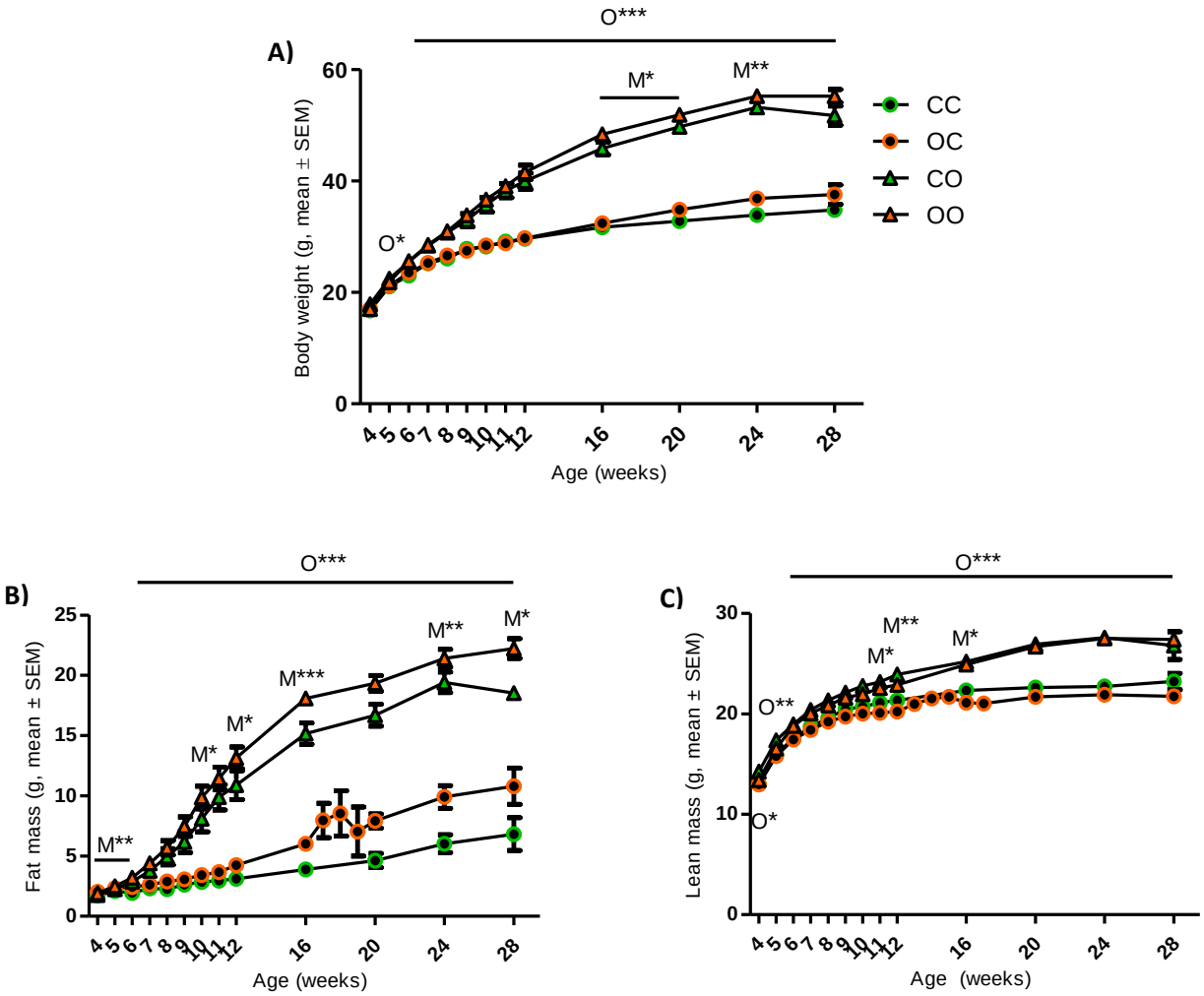

Figure 6.1. **Offspring growth and body composition up to 6 months of age. A)** Offspring body weight trajectory from 4 weeks to 6 months of age, CC>8, OC>12, CO>11, OO>12. **B)** Offspring lean mass trajectory from 4 weeks to 6 months of age. **C)** Offspring fat mass trajectory from 4 weeks to 6 months of age. CC>8, OC>9, CO>9, OO>10. 2-way ANOVA; M = effect of maternal diet, O = effect of offspring diet.* $p<0.05$, ** $p<0.001$, *** $p<0.0001$.

By 6 months of age, exposure to both maternal and offspring obesity significantly increased fed body weight. There was no significant interaction between maternal and offspring diet indicating that the effects were independent and additive. Lean mass was increased by offspring obesity only, whereas the relative lean mass was decreased by both maternal and offspring diet (consistent with adiposity being increased). There was also an interaction between maternal and offspring diet on relative lean mass indicating that offspring obesity decreased the percentage lean mass more in offspring exposed to a maternal chow diet. Fat mass and relative fat mass were significantly increased by both offspring and maternal obesity. There was no significant interaction between maternal and offspring diet on the absolute offspring fat mass, indicating that maternal and offspring obesity had independent and additive effects. The interaction between maternal and offspring diet on relative fat mass showed that offspring obesity increased the percentage fat mass more in offspring exposed to maternal obesity. These results demonstrate that the increase in 6 month offspring body weight due to maternal obesity was attributable to an increase in fat mass. These results are summarised in Table 6.2.

Table 6.2. *Offspring weight and body composition at 6 months of age.*

	CC (n<7)	**OC (n<11)**	**CO (n<7)**	**OO (n<10)**	**P value summary**
Fed body weight (g)	33.87±0.42	36.81±0.96	53.16±1	55.18±0.92	**M**** **O*****
Lean mass (g)	22.73±0.49	21.87±0.47	27.1±0.24	27.85±0.29	**O*****
Relative lean mass (%)	66.06±1.82	55.83±1.7(***)	52.15±0.93	59.92±1.16	**M**** **O***** **I***
Fat mass (g)	6.02±0.74	9.65±0.82(**)	19.42±0.86	21.41±0.74	**M**** **O*****
Relative fat mass (%)	18.53±2.03	30.47±1.48(***)	36.2±1	38.9±1.11	**M***** **O***** **I***

N numbers are presented within the table. 2 way ANOVA; M= maternal diet effect, O= offspring diet effect, I= interaction effect between maternal and offspring diets. *p<0.05, **p<0.001, ***p<0.0001. Stars in OC column indicate significance following post hoc testing relative to CC group.

Exposure to both a maternal obesogenic diet and an offspring obesogenic diet significantly increased absolute heart weight at 6 months of age (Figure 6.2.A). The maternal and offspring diet effects were additive with the OO group having the heaviest hearts (Figure 6.2.A). When heart weight was normalised to body weight, offspring weaned onto an obesogenic diet showed a significant decrease in relative heart weight (Figure 6.2.B). However, when heart weight was normalised to lean mass, exposure to maternal obesity significantly increased heart weight (Figure 6.2.C).

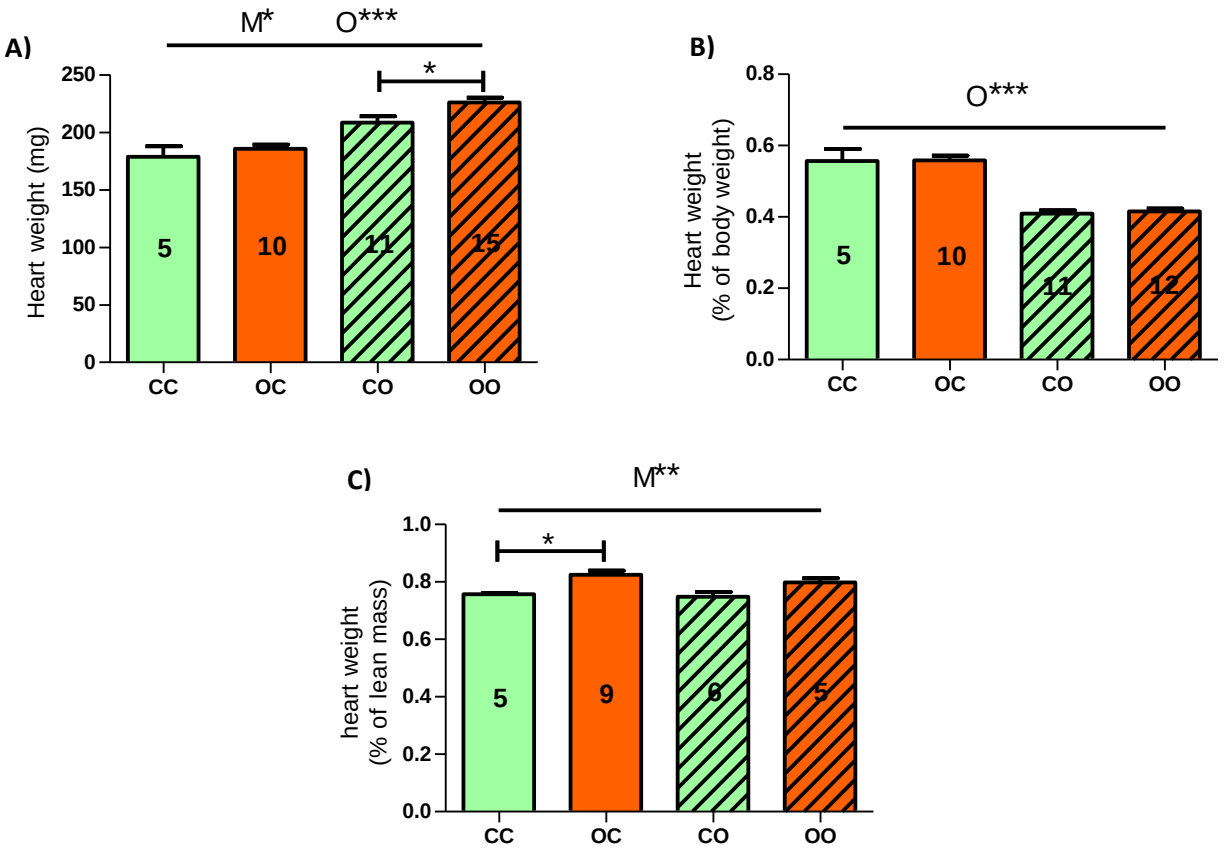

Figure 6.2. **Offspring heart weight at 6 months of age. A)** Absolute heart weight. **B)** Heart weight relative to fasted body weight. **C)** Heart weight relative to lean mass. N numbers are presented within the graphs. Green bars = offspring exposed to maternal control diet, orange bars = offspring exposed to maternal obesogenic diet, un-patterned bars = offspring exposed to control post-weaning diet, patterned bars = offspring exposed to post-weaning obesogenic diet. 2-way ANOVA; O = offspring diet effect, M = maternal diet effect. Uncapped lines indicate overall significance. Capped lines denote significance between groups following a post-hoc test. *p<0.05, **p<0.001, *** p<0.0001.

Kidney weights in 6 month old offspring mirrored those of the heart, with offspring obesity significantly increasing absolute weights (Figure 6.3.A), and significantly decreasing weights relative to fasted body weight (Figure 6.3.B). However, unlike heart weight, maternal obesity had no significant effect on either absolute kidney weight or kidney weight relative to body weight. Kidney weight normalised to lean mass was significantly increased by exposure to offspring obesity (Figure 6.3.C).

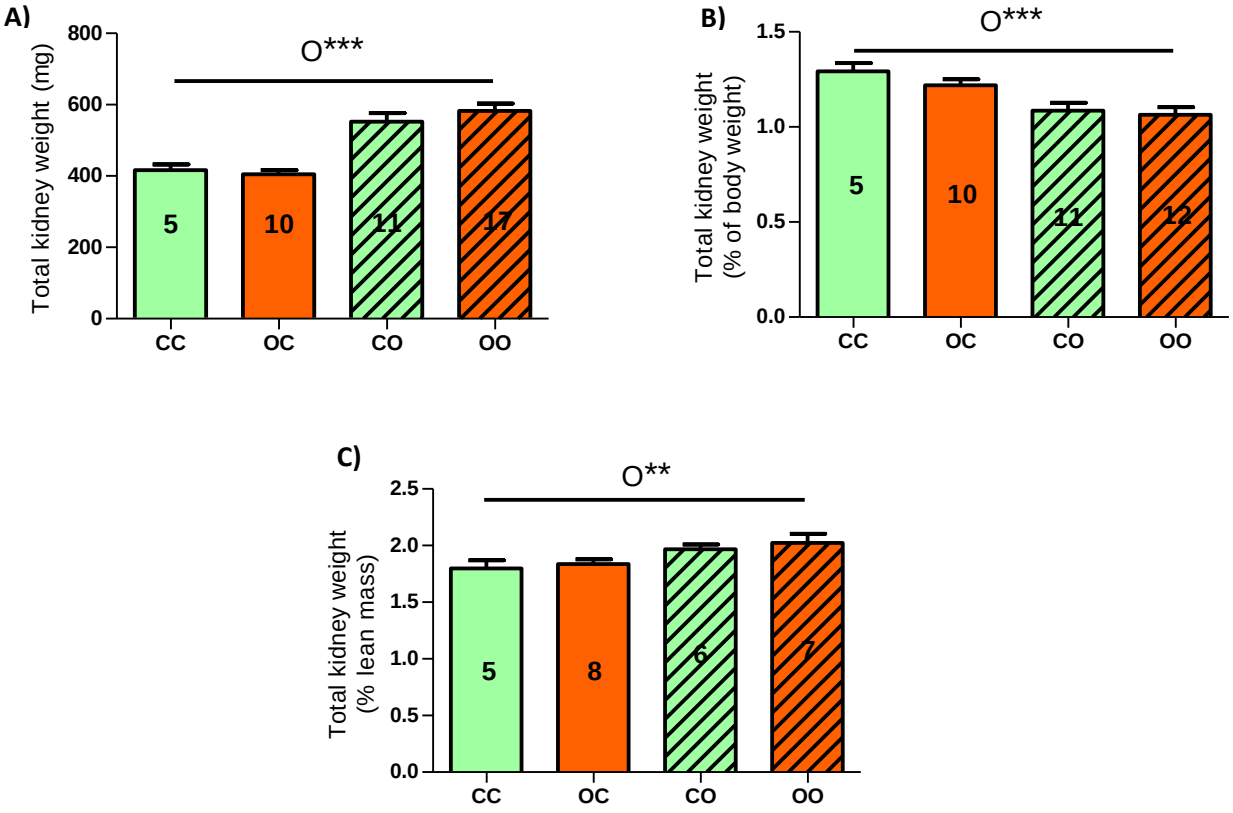

Figure 6.3. **Offspring kidney weights at 6 months of age. A)** Absolute total kidney weights. **D)** Total kidney weight relative to fasted body weight. **C)** Total kidney weight relative to lean mass. N numbers are presented within the graphs. Green bars = offspring exposed to maternal control diet, orange bars = offspring exposed to maternal obesogenic diet, un-patterned bars = offspring exposed to control post-weaning diet, patterned bars = offspring exposed to post-weaning obesogenic diet. 2-way ANOVA; O = offspring diet effect. ** $p<0.001$, *** $p<0.0001$.

Offspring obesity significantly increased serum cholesterol, LDL, and insulin and decreased triglyceride and FFA levels at 6 months of age. Exposure to maternal obesity significantly increased offspring cholesterol and LDL. There was an interaction between maternal and offspring obesity on cholesterol and LDL levels, with maternal obesity increasing levels more in offspring exposed to post-weaning obesity. There was also a significant interaction between a maternal and offspring diet on FFAs, with offspring obesity decreasing FFA levels more in offspring exposed to maternal obesity. These results are summarised in Table 6.3.

Table 6.3. *Offspring 4 hour fasted serology at 6 months of age.*

	CC (n=6)	**OC (n=10)**	**CO (n=8)**	**OO (n=9)**	**P value summary**
Cholesterol (mmol/l)	3.25±0.06	3.46±0.13	6.48±0.7	8.68±0.17 (***)	**O*** M** I***
LDL (mmol/l)	0.7±0.05	0.57±0.09	2.87±0.47	4.39±0.12 (***)	**O*** M* I****
Triglycerides (mmol/l)	1.22±0.13	1.43±0.09	1.07±0.1	1.07±0.04	**O***
FFA (µmol/l)	913±62.7	1129±51.7 (*)	877±78.2	835±24	**O** I***
Insulin (pmol/l)	241±80	310±45.8	1679±190	1943±154.6	**O***
Tail blood glucose (mmol/l)	9.7±0.7	10±0.7	9.1±0.7	9.1±0.9	

N numbers are presented within the table. 2-way ANOVA; O= offspring diet effect, M= maternal diet effect, I= Interaction effect between maternal and offspring diets. *p<0.05, **p<0.001, ***p<0.0001. Stars within offspring group columns in brackets represent significance between OC vs. CC or OO vs CO following post-hoc testing.

At 6 months of age, offspring consuming an obesogenic diet showed a small but significant increase in systolic blood pressure (p=0.012; Figure 6.4.A), whereas exposure to a maternal diet had no effect. Heart rate was not significantly influenced by either a maternal or offspring diet (Figure 6.4.B).

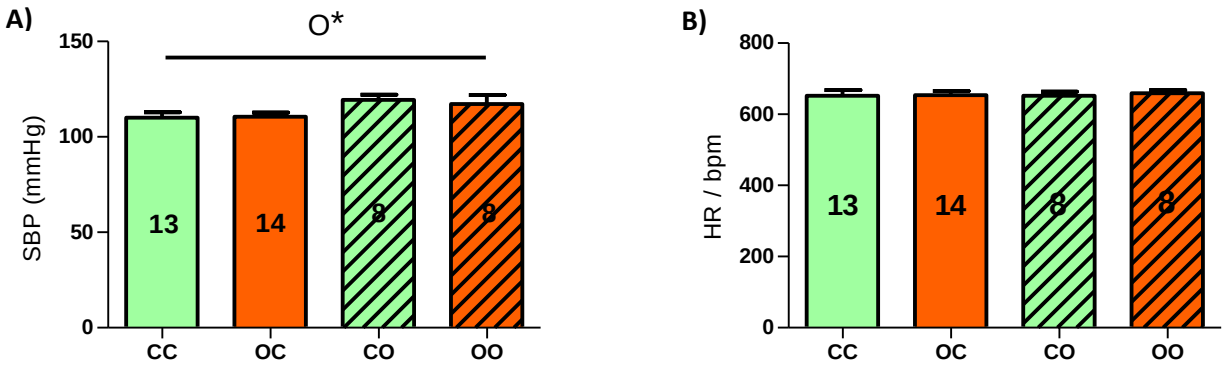

Figure 6.4. **Offspring cardiovascular function at 6 months of age. A)** Mean systolic blood pressure **B)** Mean heart rate. N numbers are presented within the graphs. Green bars = offspring exposed to maternal control diet, orange bars = offspring exposed to maternal obesogenic diet, un-patterned bars = offspring exposed to control post-weaning diet, patterned bars = offspring exposed to post-weaning obesogenic diet. 2-way ANOVA; O = offspring diet effect. * p<0.05.

At 6 months of age, an offspring obesogenic diet significantly increased medulla and cortex areas whereas maternal diet had no significant effect (Figure 6.5.A). Glomeruli density was also significantly decreased by an offspring obesogenic diet, but maternal obesity had no significant effect (Figure 6.5.B). An offspring obesogenic diet increased the mean glomerular area, whereas maternal diet had no significant effect (Figure 6.5.C).

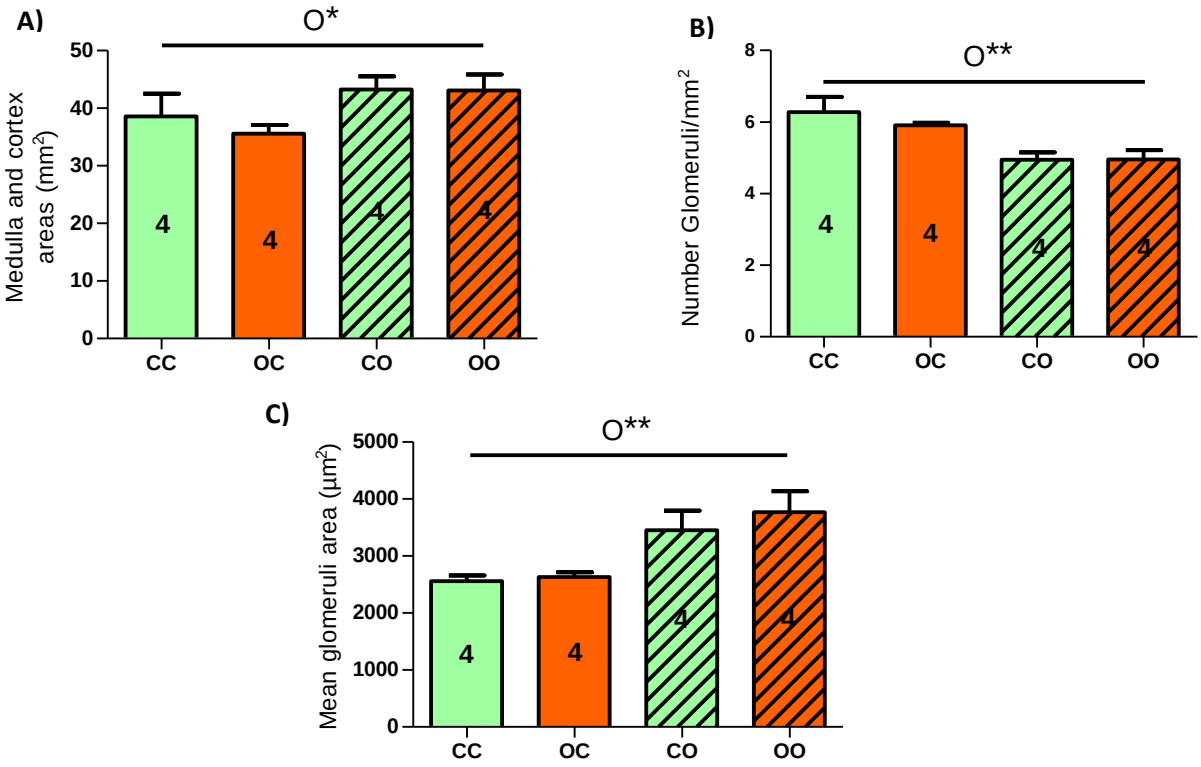

Figure 6.5. **Offspring glomerular areas at 6 months of age. A)** Medulla and cortex areas. **B)** Glomeruli density. **C)** Mean glomeruli areas. N numbers are presented within the graphs. Green bars = offspring exposed to maternal control diet, orange bars = offspring exposed to maternal obesogenic diet, un-patterned bars = offspring exposed to control post-weaning diet, patterned bars = offspring exposed to post-weaning obesogenic diet. 2-way ANOVA; O = offspring diet effect. **p*<0.05, ***p*<0.001.

At 6 months of age, serum BUN levels were unaffected by either exposure to maternal obesity or an offspring obesogenic diet (Figure 6.6.A). A maternal obesogenic diet also had no significant effect on serum cystatin C levels (Figure 6.6.B). However, cystatin C was significantly increased by an offspring obesogenic diet (Figure 6.6.B).

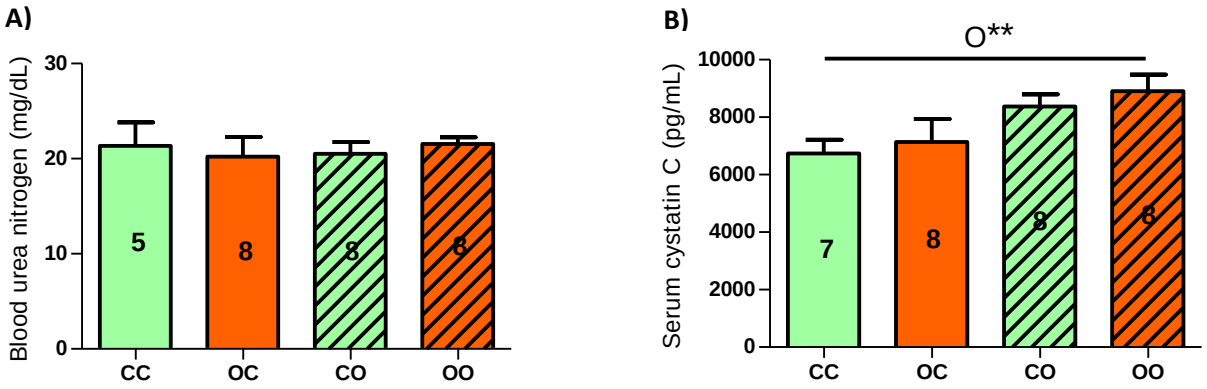

Figure 6.6. **Serum markers of renal function in 6 month old offspring. A)** Blood urea nitrogen **B)** Serum cystatin C. N numbers are presented within the graphs. Green bars = offspring exposed to maternal control diet, orange bars = offspring exposed to maternal obesogenic diet, un-patterned bars = offspring exposed to control post-weaning diet, patterned bars = offspring exposed to post-weaning obesogenic diet. 2-way ANOVA; O = offspring diet effect. ** $p<0.001$.

At 6 months of age, lipid droplets could be seen within the kidneys of all animals exposed at some time to an obesogenic diet, i.e. offspring exposed to control maternal diet and post-weaning obesogenic diet (CO), OC and OO offspring (Figure 6.7.A). An offspring obesogenic diet significantly increased intra-renal lipid content (Figure 6.7.B), whereas maternal obesity had no effect. However, there was a borderline significant interaction between a maternal and offspring diet on lipid levels ($p=0.081$), reflecting OC animals having higher lipid levels relative to offspring exposed to both a maternal and post-weaning control diet (CC), but OO animals not having higher lipid levels than CO animals (Figure 6.7.A & B). This is consistent with histological observations (see Figure 6.7.A). Lipoprotein lipase (*Lpl*) mRNA was significantly decreased by both maternal and offspring obesity, and there was an interaction between these two conditions (Figure 6.7.C).

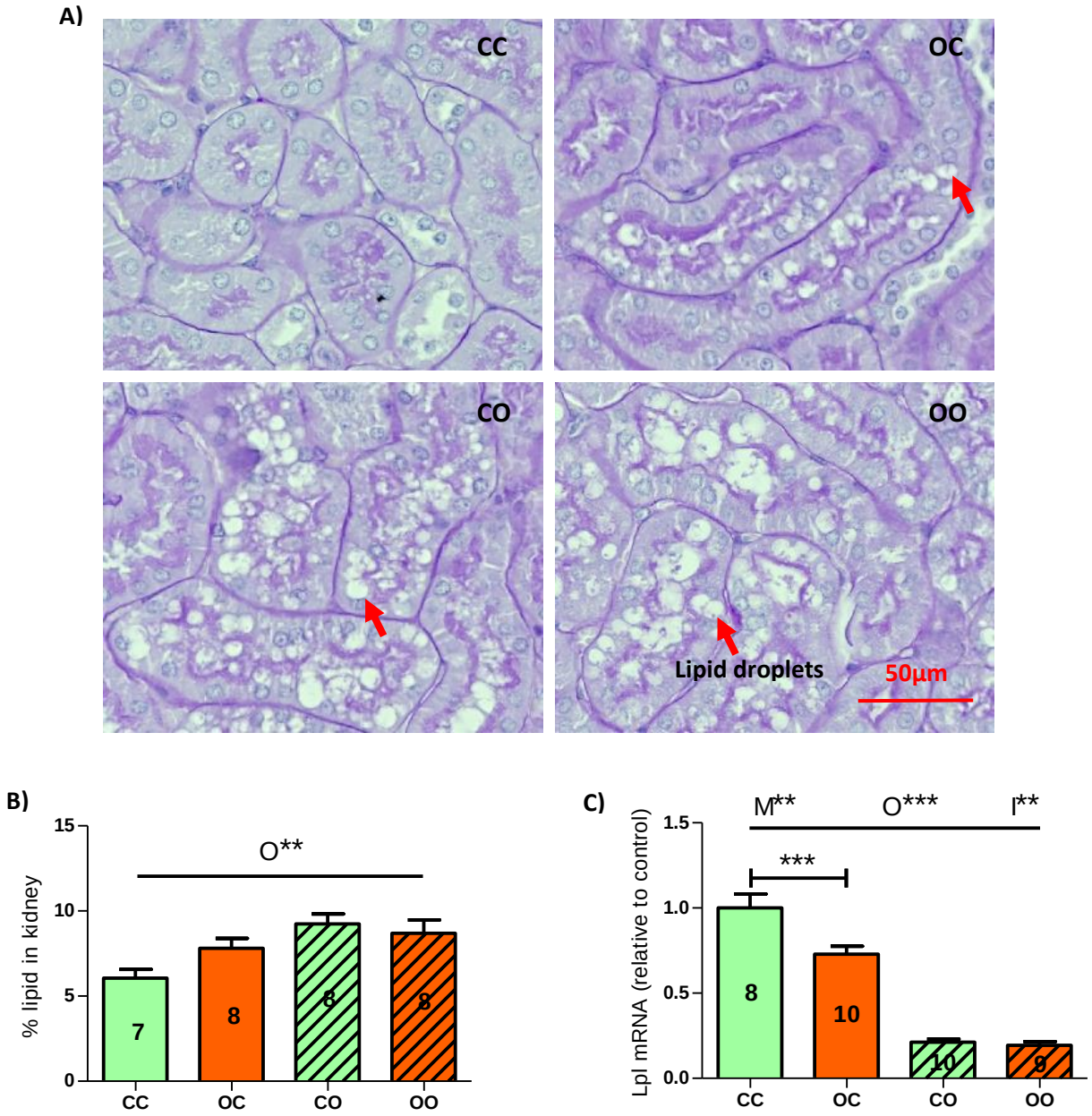

Figure 6.7. **Intra-renal lipid content in 6 month old offspring. A)** Typical PAS stained sections from 6m offspring. Red arrows denote lipid droplets (white). **B)** % lipid content in 6m renal tissue. N numbers are presented in the graph. Green bars = offspring exposed to maternal control diet, orange bars = offspring exposed to maternal obesogenic diet, un-patterned bars = offspring exposed to control post-weaning diet, patterned bars = offspring exposed to post-weaning obesogenic diet. 2-way ANOVA; M = effect of maternal diet, O = effect of offspring diet, I = interaction effect between maternal and offspring diets. Uncapped lines indicate overall significance. Capped lines denote significance between groups following a post-hoc test. ** p<0.001, *** p<0.0001.

Fn1 mRNA was significantly increased by offspring obesity within the kidneys of 6 month old males (Figure 6.8.A), but was unaffected by exposure to maternal obesity. There was a tendency for an interaction between maternal and offspring diets on *Fn1* levels (p=0.088), with OO animals showing higher levels relative to all other groups. *Mmp2* mRNA levels were however increased by both offspring and maternal obesity and the effects were additive (Figure 6.8.B). There was an interaction between maternal obesity and offspring obesity on *Casp12* mRNA (Figure 6.8.C), with expression being increased when animals were exposed to both maternal and offspring obesity. An offspring obesogenic diet also increased *Ccl2* mRNA expression (Figure 6.8.D), whereas maternal obesity had no significant impact. Similarly, *Kim1* mRNA expression was also significantly increased by an offspring diet (Figure 6.8.E) and was unaffected by maternal obesity.

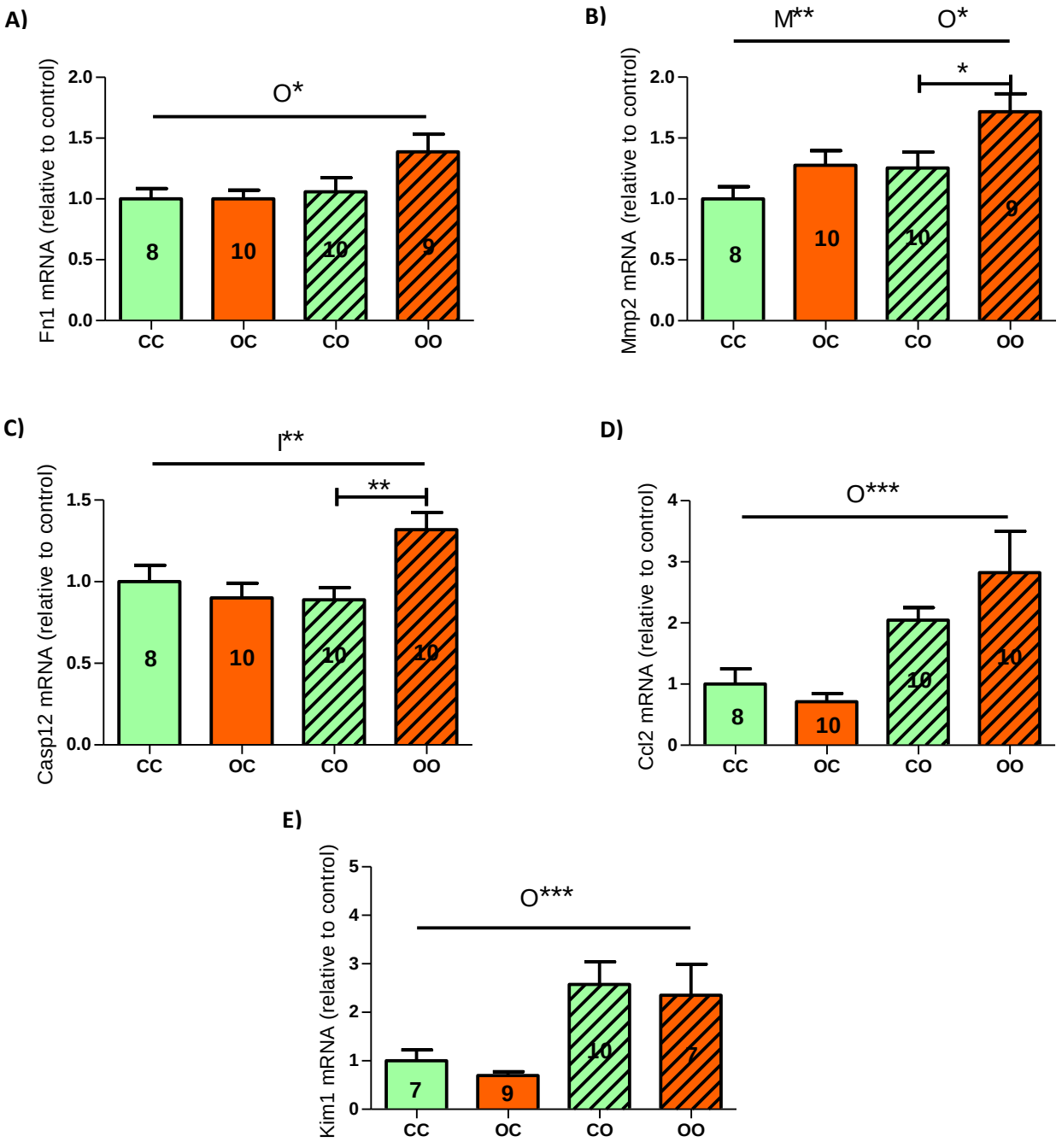

Figure 6.8. **mRNA markers of renal damage in 6 month old offspring. A)** Fibronectin. **B)** Matrix metalloproteinase 2. **C)** Caspase 12. **D)** Monocyte chemoattractant protein 1. **E)** Kidney injury molecule-1. N numbers are presented in graphs. Green bars = offspring exposed to maternal control diet, orange bars = offspring exposed to maternal obesogenic diet, un-patterned bars = offspring exposed to control post-weaning diet, patterned bars = offspring exposed to post-weaning obesogenic diet. 2-way ANOVA; M = effect of maternal diet, O = effect of offspring diet, I = interaction effect between maternal and offspring diets. Uncapped lines indicate overall significance. Capped lines denote significance between groups following a post-hoc test. * $p<0.05$, ** $p<0.001$, *** $p<0.0001$.

Exposure to maternal obesity significantly increased offspring glomerulosclerosis (Figures 6.9.A, B &C). An offspring obesogenic diet also increased glomerulosclerosis (Figures 6.9.A, B &C). The effects of maternal and offspring obesity were additive with offspring exposed to both conditions exhibiting the most overt sclerosis.

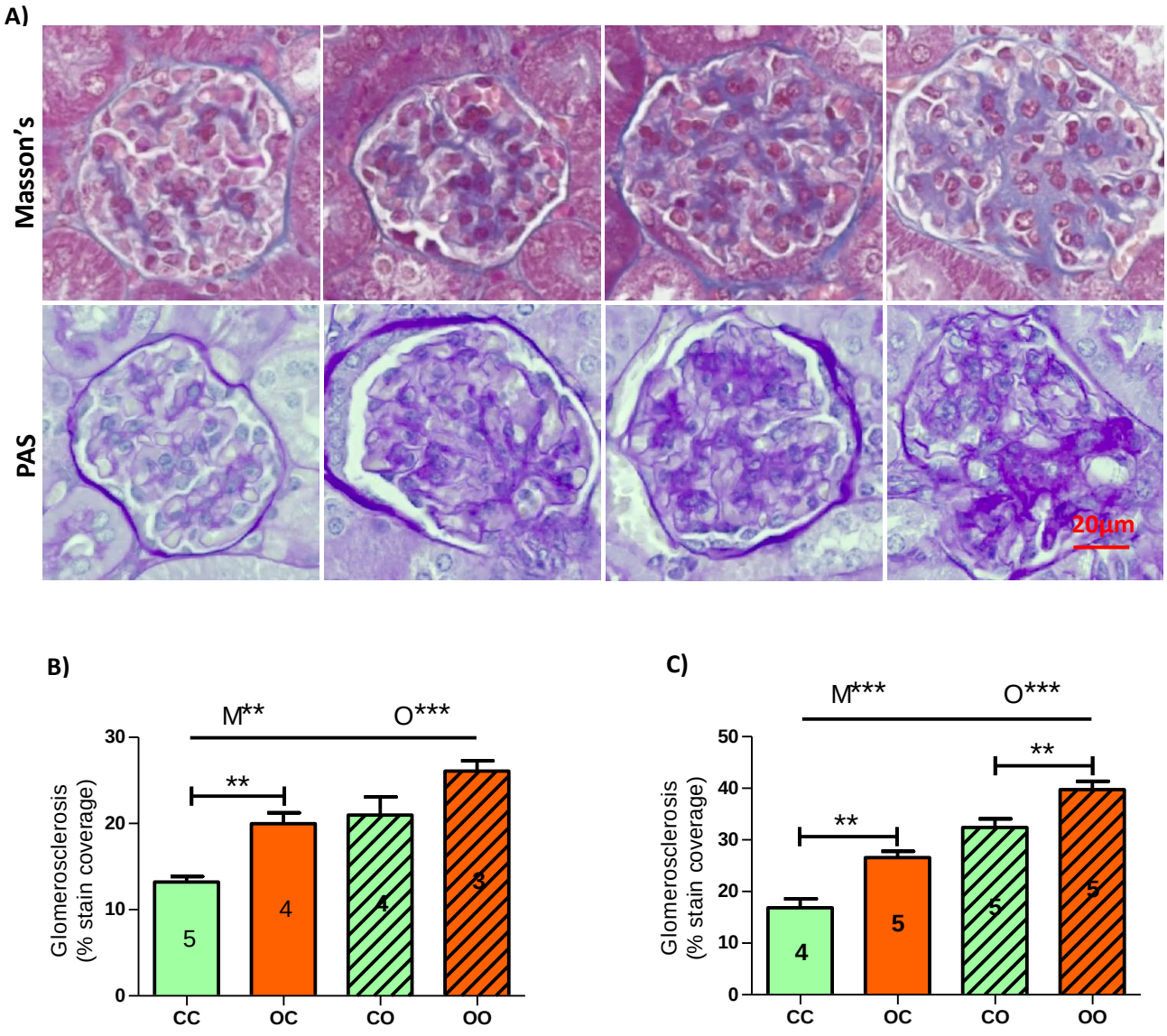

Figure 6.9. Glomerulosclerosis in 6 month old offspring. A) Masson's trichrome and PAS stained glomeruli from CC, OC, CO and OO males, blue and magenta stain respectively shows connective tissue. B) Mean % stain coverage within glomeruli following Masson's trichrome staining. C) Mean % stain coverage within glomeruli following PAS staining. Green bars = offspring exposed to maternal control diet, orange bars = offspring exposed to maternal obesogenic diet, un-patterned bars = offspring exposed to control post-weaning diet, patterned bars = offspring exposed to post-weaning obesogenic diet. 2 way ANOVA; M= effect of maternal diet, O= effect of offspring diet. Uncapped lines indicate overall significance. Capped lines indicate significance between groups following a Bonferroni post-hoc test. ** $p<0.01$ *** $p<0.001$.

Maternal obesity had a significant effect on tubulointerstitial fibrosis as assessed by both masson's trichrome staining and PAS staining (Figures 6.10.A, B & C). PAS staining showed that offspring obesity had a significant effect on tubulointerstitial fibrosis (Figures 6.10.A & C), however masson's trichrome staining showed no effect of offspring diet (Figures 6.10 A &B).

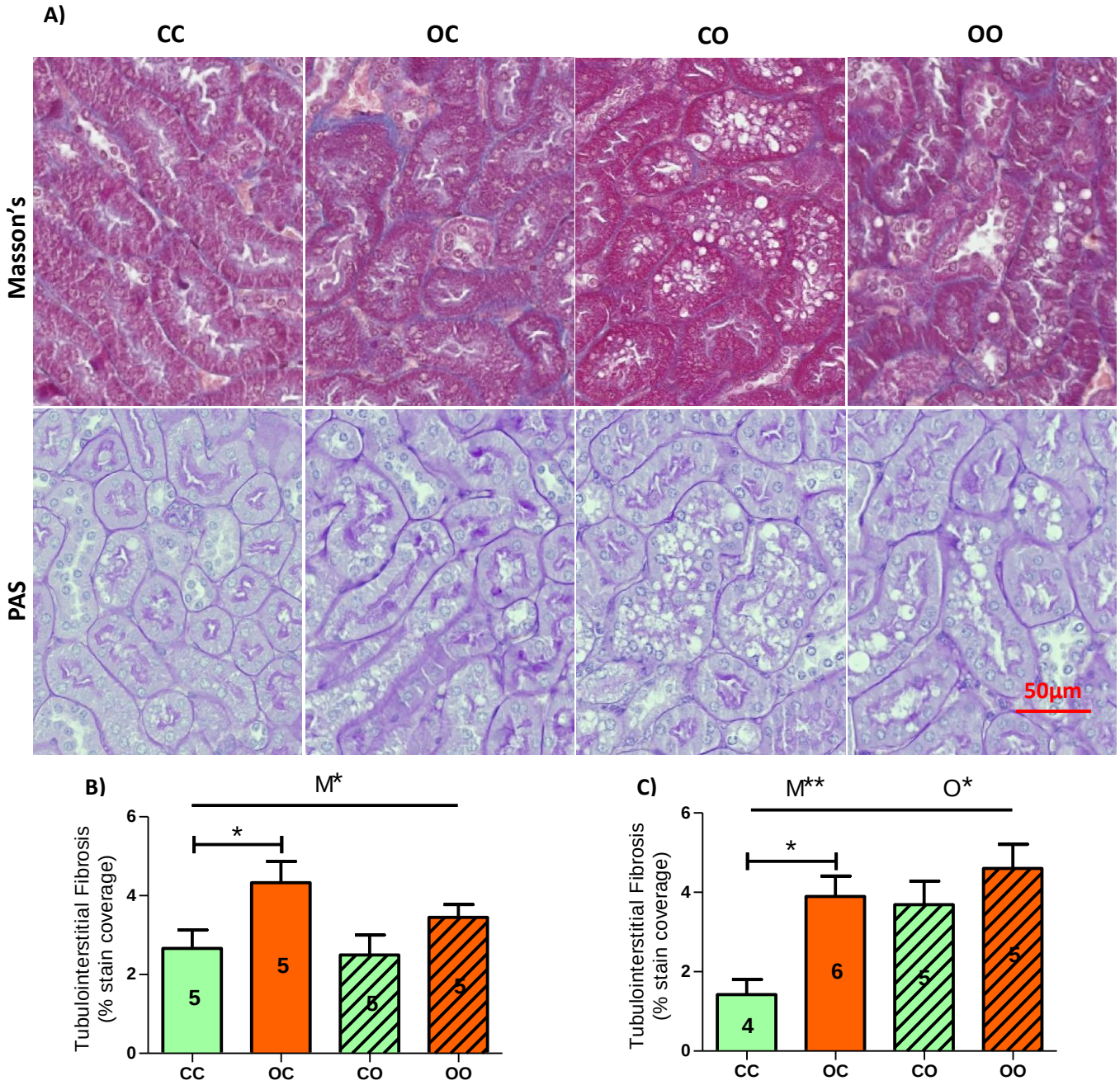

Figure 6.10. Tubulointerstitial fibrosis in 6 month old offspring. A) Masson's trichrome and PAS stained cortex from CC, OC, CO and OO offspring, blue and magenta stain show connective tissue respectively. B) Mean % stain coverage within the cortex (excluding glomeruli) following Masson's trichrome staining. C) Mean % stain coverage within the cortex (excluding glomeruli) following PAS staining. Green bars = offspring exposed to maternal control diet, orange bars = offspring exposed to maternal obesogenic diet, un-patterned bars = offspring exposed to control post-weaning diet, patterned bars = offspring exposed to post-weaning obesogenic diet.2 way ANOVA; M= effect of maternal diet, O= effect of offspring diet. Uncapped lines indicate overall significance. Capped lines indicate significance between groups following a Bonferroni post-hoc test. *p<0.05, **p<0.001.

6.5 Discussion

The aim of this chapter was to assess the impact of a maternal obesogenic diet in combination with ageing and/or a post-weaning obesogenic diet on offspring blood pressure, renal function and renal damage.

6.5.1 Offspring phenotype

A post-weaning obesogenic diet increased offspring weight at all ages measured and increased offspring fat mass from 6 weeks of age onwards. Importantly maternal obesity also led to an increase in offspring weight from 16 weeks of age onwards. An increase in fat mass was seen in offspring of obese dams from around 10 -12 weeks until the end of the study with no consistent effect on lean mass. Therefore the increase in offspring weight due to maternal obesity can be attributed to increased adiposity. This is consistent with results from another laboratory using an identical mouse model of maternal diet induced obesity, where exposed offspring showed increased inguinal fat mass at 6 months of age (Samuelsson et al. 2008). Since in the aforementioned study, offspring hyperphagia was observed in offspring of obese dams before significant weight or fat mass gain, this suggests that the offspring exposed to maternal obesity in the current study may also show an increase in weight and fat mass due to an increase in food intake. Indeed, as discussed in section 6.1.3, there is evidence demonstrating that a maternal obesogenic diet leads to a preference for sugary and fatty foods in the offspring as well as a tendency for the offspring to eat more. The additive effect of maternal and offspring obesity on offspring fat mass in the current study is also consistent with a study demonstrating that male rats exposed to a maternal high fat and fructose diet and fed the same diet postnatally had 30% more visceral fat than rats fed a postnatal high fat and fructose diet alone (Jackson et al. 2012).

Heart weight was increased by a post-weaning obesogenic diet in 6 month old offspring. This is consistent with other animal models and epidemiological studies showing that obesity increases heart weight and causes cardiac hypertrophy (Abel et al. 2008). An increase in cardiac hypertrophy and weight in obesity is adaptive in order to cope with the increased demand placed on the heart by an increased blood volume, associated with the elevated body mass, and blood pressure. Although adaptive at first, these features are thought to be precursors to more overt forms of cardiac dysfunction (Abel et al. 2008). Importantly, maternal obesity also increased absolute heart weight and heart weight normalised to lean mass in 6 month old offspring. This is consistent with findings at 3 and 8 weeks, before there was an increase in offspring weight (see chapters 4 and 5 respectively), and

likely associated pathological cardiac hypertrophy as seen at younger time points and discussed in chapter 5, section 5.5.1. These findings suggest that offspring exposed to maternal obesity have a greater risk of cardiac dysfunction at 6 months of age, and that this risk is exacerbated when offspring are challenged with a post-weaning obesogenic diet.

Offspring kidney weight at 6 months was also increased by a post-weaning obesogenic diet. Similarly to the heart, neurohormonal and haemodynamic changes associated with obesity challenge the kidney leading to hyperfiltration, which is thought to promote an increase in kidney weight as well as an increase in the Bowman's space and tubular lumen, mesangial matrix expansion and thickening of the glomerular and tubular basement membranes (Henegar et al. 2001; D'Agati et al. 2016). Similarly to the heart, these changes often precede more overt forms of damage such as renal fibrosis, which was observed in offspring exposed both to maternal obesity and offspring obesity in the current study.

A post-weaning obesogenic diet increased offspring serum cholesterol, insulin levels and decreased FFA levels. In addition to the increase in weight, fat mass and blood pressure also observed in offspring exposed to a post-weaning obesogenic diet in the current study, this blood profile is consistent with a metabolic syndrome phenotype (Alberti et al. 2005). Importantly, exposure to maternal obesity also increased offspring cholesterol and LDL levels at 6 months of age, with the effect being most pronounced in offspring also exposed to a post-weaning obesogenic diet. This is in contrast with offspring exposed to maternal obesity at 8 weeks of age (see chapter 5), and therefore suggests that ageing and/or a later life obesogenic challenge intensifies adversities in lipid metabolism programmed by exposure to maternal obesity. This observation is consistent with epidemiological studies showing that exposure to maternal obesity is associated with long-term health consequences in the offspring including dyslipidaemia (O'Reilly & Reynolds 2013), and suggests that offspring exposed to maternal obesity may be more at risk of CVD. In contrast to 8 week old offspring (see chapter 5), maternal diet had no significant effect on offspring insulin levels at 6 months of age. This is most likely due to the fact that offspring obesity had such a strong effect on insulin levels, that this obscured any effects of the maternal diet. Indeed, insulin levels increased from 8 weeks to 6 months of age in both CC and OC offspring, and in 6 month animals the highest insulin levels were seen in offspring exposed to both maternal and post-weaning obesity. The lack of a significant maternal dietary effect on offspring insulin levels at 6 months of age may therefore be due to insufficient power of the statistical test used, especially since the CC group had a low sample size relative to other groups.

The current data indicate that as expected, a post-weaning obesogenic diet resulted in characteristic metabolic syndrome in exposed offspring at 6 months of age. Notably, maternal obesity appeared to have an additive effect on characteristics including fat mass, heart weight and cholesterol levels,

indicating that an offspring obesogenic diet may accentuate the adverse impacts of a maternal obesogenic environment.

6.5.2 Blood pressure

Blood pressure was increased by an offspring obesogenic diet. This is consistent with other models demonstrating increased blood pressure in animals exposed to a post-weaning high fat, high fructose or western diet (Sharma et al. 2007; Zhang et al. 2008; Inoue et al. 2012; Vileigas et al. 2016; Tain et al. 2017). The relationship between obesity and hypertension is well established and is thought to arise from an array of obesity related factors including activation of the sympathetic nervous system, alterations in renal function, changes in neurohormones and vascular endothelial dysfunction (Kotsis et al. 2010). Maternal diet had no significant effect on offspring blood pressure at 6 months of age, despite increasing offspring blood pressure at 8 weeks of age (see chapter 5). This observation is also in contrast with findings from an identical mouse model of maternal diet-induced obesity, and with a rat model of maternal diet induced obesity, which demonstrated increased offspring blood pressure at 6 months of age (Samuelsson et al. 2008; Samuelsson et al. 2016). These discrepancies might be due to differences in the timing and methods used for blood pressure measurement. Samuelsson and colleagues showed using radiotelemetry that offspring exposed to maternal obesity demonstrated a significant increase in blood pressure compared to control offspring only during the dark phase or the "active phase" for mice. A rise in dark phase but not light phase blood pressure in offspring exposed to maternal obesity would be consistent with changes in factors under circadian control. The hypothalamic–pituitary–adrenal axis (HPA) axis and cortisol action are known to be highest during the morning in humans consistent with promoting wakefulness (Morris et al. 2012). In mice, this would be equivalent to the beginning of the dark phase, which in accordance with the time blood pressure was seen to increase in mice exposed to maternal obesity (Samuelsson et al. 2008). In the current study, we measured blood pressure before the dark phase (between 3 and 4pm) raising the possibility that changes in blood pressure between offspring exposed to maternal obesity and offspring exposed to a maternal control diet were undetected. Alternatively, the lack of an effect of maternal obesity on offspring blood pressure at 6 months of age could just reflect a lack of a stress related response to the blood pressure protocol in these animals. At 8 weeks of age, where blood pressure was measured at a similar time of the day and maternal obesity had a significant effect, offspring underwent the tail-cuff blood pressure procedure for the first time. Conversely, by 6 months of age, offspring had already experienced the protocol previously in young adulthood and therefore may have been more acclimatised to the procedure. This may have decreased any contributory effects of programmed

stress to blood pressure in 6 month old offspring, removing the effect of maternal obesity. In support of this, as discussed in chapter 5, section 5.5.2, animal models of maternal obesity have shown high offspring blood pressure together with alterations in offspring HPA axis activity leading to altered corticosterone levels and anxiety like phenotypes. The current findings together with previous findings highlight the need to measure blood pressure during the night in offspring exposed to maternal obesity and to also explore the contribution of stress to blood pressure regulation.

Heart rate was unaffected in offspring exposed to a post-weaning obesogenic diet. This is consistent with rodent models of high fat feeding which have demonstrated an increase in blood pressure but not in heart rate in obese animals (Williams et al. 2003; Dubinion et al. 2010; Vileigas et al. 2016). Dubinion and colleagues were able to show a reduction in blood pressure but not heart rate in obese animals following melanocortin receptor 3/4 (MC3/4R) antagonism (Dubinion et al. 2010). This demonstrates a role for the central sympathetic nervous system MC3/4R in obesity related hypertension independent of HR, and therefore suggests offspring exposed to post-weaning obesity in the current study may also demonstrate central sympathetic nervous system dependent hypertension. Maternal diet also had no effect on offspring heart rate at 6 months of age. This is in contrast to findings at 8 weeks of age. Like with blood pressure, this could be due to the fact that measurements were taken at a different time of the day between age groups.

6.5.3 Renal morphological phenotype

Renal medulla and cortex areas were increased in offspring exposed to a post-weaning obesogenic diet. This is consistent with the increased total renal weight also observed in these animals, and with other animal models of high fat diet induced obesity (Henegar et al. 2001; Altunkaynak et al. 2008). In the latter study, the authors suggested that the increased cortex and medulla volume observed in high fat diet exposed rats was due to the dilatation of renal blood vessels and connective tissue expansion also seen in these animals (Altunkaynak et al. 2008). Other studies have demonstrated increased kidney weight in high fat fed rodents, together with tubular dilatation and vacuolisation (Armitage et al. 2005; Glastras, Chen, et al. 2016). Accordingly, in obese patients vacuolation of mesangial and tubular epithelial cells has been observed, as well as enlargement of the proximal tubule lumen attributed to increases in GFR (D'Agati et al. 2016). Increased tubular volume has also been correlated with BMI, GFR and hypertension in humans (Hoy et al. 2014). An increase in renal fibrosis and vacuolation was observed in offspring exposed to post-weaning obesity in the current study, consistent with the increased medulla and cortex area. Going forward, it would be interesting to assess the potential contribution of vessel and tubular dilatation to this phenotype.

Glomerular density was reduced in offspring exposed to a post-weaning obesogenic diet. A reduced glomerular density is an established feature of obesity related glomerulopathy (a pathology featuring proteinuria, glomerulomegaly, progressive glomerulosclerosis and renal functional decline) in humans, however whether this is due to a decreased nephron endowment or tubular hypertrophy in these patients is yet to be established (D'Agati et al. 2016). In the current study, it is likely that the decreased glomerular density reflects the increase in medulla and cortex area also observed in offspring exposed to a post-weaning obesogenic diet. In support of this, a high fat and sucrose diet in minipigs led to a decreased glomerular density together with tubular swelling and vacuolation (Li et al. 2015). A decrease in glomerular density was also seen in combination with an increase in cortex and medulla volumes in a rat model of high fat feeding (Altunkaynak et al. 2008). However, in this study, the mean glomerular volume was also decreased together with an increase in the mean volume of the Bowman's capsule, suggestive of glomerular atrophy. Glomerular atrophy eventually leads to obsolescence (the collapse of the glomerular tuft). Although mean glomerular area was increased in mice exposed to a post-weaning obesogenic diet in the current study, this is not incompatible with the notion of a decreased glomerular density due to glomerular loss. In fact in hypertensive nephropathy, glomerular hypertrophy is thought to precede glomerulosclerosis leading to obsolescence (Hughson et al. 2014), suggesting that both features could be found together within damaged kidneys. Alternatively, glomerular loss could promote adaptive hypertrophy of the remaining glomeruli to cope with the functional demand (López-Novoa et al. 2011; Hoy et al. 2014), providing another explanation for the potential presence of both hypertrophic and atrophic/obsolescent glomeruli within diseased kidneys. In the future it would therefore be interesting to assess whether exposure to maternal obesity and/or a post-weaning obesogenic diet promotes glomerular atrophy or obsolescence in offspring at 6 months of age in the current model, especially since these offspring demonstrate hypertension and glomerulosclerosis, factors known to promote obsolescence.

As mentioned above, glomerular areas were increased in offspring exposed to a post-weaning obesogenic diet. Glomerular hypertrophy is an established feature of obesity in humans along with mesangial expansion, matrix accumulation and a decreased density of effaced, hypertrophied podocytes (Rea et al. 2006; Goumenos et al. 2009; D'Agati et al. 2016). Several animal models including high fat diet induced obesity in dogs (Henegar et al. 2001) and mice (Deji et al. 2009; Kuwahara et al. 2016), high fat and sucrose induced obesity in minipigs (Li et al. 2015), and genetic obesity in rats (Kubota et al. 2016; Devassy et al. 2017) have also lead to glomerular hypertrophy, consistent with the current study. The increased glomerular area in offspring exposed to a post-weaning obesogenic diet is also in accordance with the raised blood pressure in these offspring.

Glomerular hypertrophy is a central feature of obesity driven hypertensive nephropathy and is thought to arise due to an increase in intra-glomerular pressure and hyperfiltration (De Vries et al. 2014; D'Agati et al. 2016). In the current study, serum insulin levels also correlated with glomerular area (data not shown). Insulin has an important role within the kidney and the insulin receptor is found in all the cells in the glomerulus as well as throughout the entire length of the tubule. Insulin promotes local renal vasodilation leading to increased GFR (Hale & Coward 2013). Conversely, when cells such as the mesangial cells and podocytes become insulin resistant, this promotes mesangial hypertrophy and matrix deposition (Artunc et al. 2016). These characteristics are consistent with the increase in glomerular area observed in offspring exposed to a post-weaning obesogenic diet. Importantly, glomerular hypertrophy precedes glomerulosclerosis (Fogo et al. 1990; Hughson et al. 2014), which was also observed in obese offspring in the current study.

Whereas glomerular areas were increased in 3 week and 8 week old offspring exposed to maternal obesity, this effect was no longer observed in 6 month old animals. In fact 6 month old OC offspring showed a slight decrease in mean glomerular area relative to 8 week old OC offspring. However, these offspring showed an increase in glomerulosclerosis despite lacking evidence of hypertrophy. Glomerulosclerosis, if preceded by hyperfiltration, can occur following glomerular expansion. Conversely, a decrease in glomerular perfusion can lead to glomerulosclerosis through atrophy and obsolescence (Hughson et al. 2014). As discussed above, it would therefore be useful to assess the Bowman's space area relative to glomerular tuft area in offspring exposed to maternal obesity at 6 months of age to see whether atrophy could account for the reduced glomerular area observed.

6.5.4 Indicators of renal function

BUN was unaffected by exposure to either maternal or post-weaning obesity. However cystatin C was increased in the serum of offspring exposed to post-weaning obesity. The discrepancy in BUN and Cystatin C results is not surprising given that cystatin C has generally been shown to be more sensitive to renal damage than BUN and that each is influenced by different factors. In another model of diet-induced obesity in C57BL6 mice, BUN levels were similar between control and obese mice at 6 months of age and an increase was only observed in the obese group by 9 months of age. Furthermore, glomerular mesangial cell hyperplasia was observed by 6 months of age in obese mice (Hoffler et al. 2009), indicating that BUN is a late biomarker of renal impairment. In support of this, another C57BL6 mouse model showed that 12 month old male mice fed a high fat diet displayed many indicators of renal damage including increased immune cell infiltration, collagen protein expression, urinary albumin and neutrophil gelatinase-associated lipocalin (a protein secreted upon kidney injury), but no

changes in BUN levels were seen (van der Heijden et al. 2015). Conversely, there is evidence that cystatin C is more sensitive to renal damage than traditional markers. In humans, cystatin C has been shown to be superior to creatinine as a marker of GFR (Coll et al. 2000; Dharnidharka et al. 2002). However, it should be noted that cystatin C is not a perfect measure of GFR; it is known to be associated with other factors including serum glucose, diabetes and blood pressure which limit its ability to accurately predict GFR (Stevens et al. 2009). It has been suggested that an equation could be used to better predict GFR from cystatin C, taking into account serum lipids and obesity, but paradoxically this would reduce power to predict cardiovascular disease risk (Rule & Lieske 2011). In the future it would be useful to employ a more accurate measure of GFR such as inulin in 6 month old mice exposed to an obesogenic environment. This would illuminate whether cystatin C levels are raised in these animals due to a reduction in GFR or due to non-renal cardiovascular risk factors. Overall, the increase in cystatin C but not in BUN levels in 6 month old offspring exposed to a post-weaning obesogenic diet is indicative of a mild to moderate impairment in renal function in these animals. This would be consistent with the renal morphological changes and indicators of renal damage also observed in these offspring.

6.5.5 Renal damage

6.5.5.1 Lipid deposition

A post-weaning obesogenic diet was associated with a significant increase in intra-renal lipid deposition. Although maternal diet had no significant effect, lipid droplets were observed in animals exposed to maternal obesity, with OC animals showing considerably higher levels of intra-renal lipid than control animals. These results are consistent with other animal models showing that a high fat diet or high fat and sucrose diet led to increased renal lipid deposition and tubular vacuolation (Jiang et al. 2005; Deji et al. 2009; Ma et al. 2011; Declèves et al. 2014; Mount et al. 2015; Li et al. 2015; Glastras, Chen, et al. 2016), and with a rat model demonstrating that a maternal high fat diet and/or post-weaning high fat diet increased intra-renal triglyceride deposition in exposed offspring (Chowdhury et al. 2017). The increased lipid deposition levels in animals in the current study also correlated with insulin levels (data not shown). Consistently, ectopic lipid levels have been shown to correlate more strongly with insulin resistance than with any of the commonly used indicators of obesity such as BMI or waist circumference (D'Agati et al. 2016). Ectopic lipid deposition is thought to lead to insulin resistance in the podocytes leading to podocyte apoptosis, and hypertrophy of remaining podocytes. This undermines the structure of glomerular loop and leads to glomerulosclerosis (De Vries et al. 2014). Renal lipid deposition has also been shown to be associated

with obesity-related glomerulopathy, hyperfiltration, renal cell maladaptation, albuminuria, and tubular interstitial injury and fibrosis in various experimental and human biopsy studies (De Vries et al. 2014). These observations are consistent with the changes in renal morphology, glomerulosclerosis and tubulointerstitial fibrosis also observed in offspring exposed to an obesogenic environment in the current study.

A decrease in lipoprotein lipase (*Lpl*) mRNA expression was also observed in animals exposed both to a maternal and post-weaning obesogenic diet. This is in accordance with the increased intra-renal lipid deposition observed in these animals. LPL is involved in hydrolysing circulating lipids. In mice, it is located in the renal tubules and capillaries surrounding the tubules. This is most likely due to the fact that tubular epithelial cells consume a lot of energy and derive this energy mainly from fatty acids (Li et al. 2012). In the mouse, a decreased expression of renal LPL has been associated with increased intra-cellular triglyceride accumulation in proximal tubules (Li et al. 2012). Accordingly, another study also showed an increase in renal triglyceride accumulation and a decrease in renal *Lpl* expression in rats exposed to a high fat diet, and showed that other genes involved in lipid metabolism were dysregulated by either a maternal or post-weaning high fat diet (Chowdhury et al. 2017). Interestingly, in the aforementioned study kidney injury molecule 1 was also up-regulated by a high fat diet, consistent with offspring exposed to post-weaning obesity in the current study. A study in minipigs also demonstrated that a high fat and sucrose diet lead to renal lipid accumulation and alterations in genes relating to lipid storage including an increase in *Srebp1* (Li et al. 2015), which when down-regulated, has been shown to prevent lipid accumulation and CKD (De Vries et al. 2014). Importantly, LPL activity has been reported to be reduced in CKD in humans (Ruge et al. 2004). Together, the evidence discussed suggests that by 6 months of age offspring exposed to a maternal obesogenic and/or post-weaning obesogenic diet show evidence of lipid metabolism dysregulation leading to ectopic fat deposition, which is a central feature of obesity related renal disease.

6.5.5.2 Gene expression

Fn1 mRNA expression was increased by a post-weaning diet, with the highest levels being observed in OO offspring. Fibronectin is a glycoprotein of the extracellular matrix. Within the kidney it is found in the extracellular matrix of mesangial cells, and in the basement membranes of the glomerulus and tubular epithelial cells (Dixon & Burns 1982). Increased levels therefore reflect mesangial cell expansion and basement membrane thickening. An increase in *Fn1* has been shown to occur within the kidney once there is sustained injury and the kidney starts synthesising its own fibronectin rather than sequestering it from plasma (Goyal & Wiggins 1991). In rodents, intra-renal fibronectin protein expression has been shown to be increased by a post-weaning high fat diet (Jiang et al. 2005) and by

exposure to maternal obesity (Glastras et al. 2015). Furthermore, mice exposed to maternal obesity had exacerbated levels when challenged with postnatal diabetes (Glastras, Tsang, et al. 2016). These findings are consistent with the current findings.

Mmp2 mRNA levels were increased by both a maternal and post-weaning obesogenic diet, with the highest levels observed in OO animals. MMP-2 is a collagenase able to degrade collagens, gelatins, fibronectin and laminin (Lenz et al. 2000). Therefore, it was previously thought to be anti-fibrotic. However, MMP-2 has been shown to directly increase macrophage infiltration (Nishida et al. 2007), and to play a key role in the epithelial to mesenchymal cell transition (EMT) in tubule cells leading to fibrosis (Zhao et al. 2013). Accordingly in a mouse model of obstructive nephropathy, MMP-2 knockout mice showed ameliorated fibrosis and a decrease in EMT markers and macrophage infiltration (Du et al. 2012). Serum levels of MMP-2 also correlate with proteinuria and reduced renal function in patients with CKD (Zhao et al. 2013). An increase in *Mmp2* has also been observed in mice fed a high fat diet (Niu et al. 2016), consistent with the current findings.

Caspase 12 mRNA was elevated in OO offspring. Caspase 12 mediates apoptosis induced specifically by endoplasmic reticulum (ER) stress (Nakagawa et al. 2000). ER stress is an important feature in tubular epithelial cells in patients with diabetic nephropathy, and is known to be stimulated by glucose, fatty acids, albumin and oxidative stress (Lindenmeyer et al. 2008). Obese Zucker rats also showed increased activation of the ER stress signalling pathway including an increase in caspase 12, consistent with the current findings (Wang et al. 2014). Interestingly, the authors of this study showed that reducing renal lipid deposition led to a decrease in ER stress. This suggests that the lipid deposition observed in offspring exposed to obesity in the current study could be promoting ER stress driven apoptosis.

An offspring obesogenic diet increased *ccl2* mRNA levels, with OO offspring showing the highest levels. An increase in monocyte chemoattractant protein is associated with diabetic nephropathy and promotes macrophage infiltration mainly into the glomerulus and interstitium (Chow et al. 2006). MCP-1 knockout mice were protected from macrophage infiltration and fibrosis (Chow et al. 2006). Consistent with the current study, mice exposed to maternal obesity (Glastras et al. 2015), a post-weaning obesogenic diet (Glastras et al. 2017) or diabetes (Glastras, Tsang, et al. 2016) have shown an increase in renal MCP-1 levels.

Kim1 was increased in offspring exposed to a post-weaning obesogenic diet. KIM-1 is a transmembrane receptor on tubular epithelial cells that recognises apoptotic cells and directs them to lysosomes (Vaidya et al. 2010). It is conserved across zebrafish, rodents, dogs, primates and humans, and within these species mRNA levels are elevated more than any other known gene after

renal injury (Vaidya et al. 2010). As such, KIM-1 has been shown to outperform serum creatinine, BUN and urinary NAG as a diagnostic marker in multiple rat models of kidney injury (Vaidya et al. 2010). Accordingly, in humans KIM-1 protein expression in proximal tubular cells correlated with tubulointerstitial fibrosis and inflammation (Parikh et al. 2007). Furthermore, overexpression of *Kim1* in the absence of renal injury in mice led to spontaneous interstitial kidney fibrosis and inflammation leading to renal failure (Humphreys et al. 2013). The authors of this study also demonstrated *in vitro* that KIM-1 expression led to MCP-1 production consistent with the current findings.

Overall, the renal gene expression profile of offspring in the current study suggests that a post-weaning obesogenic diet promotes renal fibrosis and inflammation, and that exposure to maternal obesity may exacerbate some markers of damage. This is consistent with the increased glomerulosclerosis and tubulointerstitial fibrosis seen in these animals.

6.5.5.3 Fibrosis

Both maternal obesity and offspring obesity independently increased offspring glomerulosclerosis and there was an additive effect between these conditions resulting in OO offspring showing the most extensive fibrosis. Tubulointerstitial fibrosis was also increased by both a maternal and offspring obesogenic diet. This is in accordance with other studies demonstrating that male mice exposed to either a maternal high fat diet (Tain et al. 2017) or high fat and fructose diet (Jackson et al. 2012) showed more exaggerated glomerulosclerosis and tubulointerstitial fibrosis if they were weaned onto the same diet themselves. Likewise, by 32 weeks of age mice exposed to a maternal high fat diet exhibited increased glomerulosclerosis and tubular interstitial injury which was accentuated by exposure to STZ administered at 8 weeks of age (Glastras, Tsang, et al. 2016). The increased glomerulosclerosis and tubulointerstitial is also in accordance with several other characteristics observed in offspring exposed to a maternal obesogenic or offspring obesogenic diet in the current study including altered renal morphology, intra-renal fat deposition and altered gene expression.

The current study together with previous studies has demonstrated that maternal obesity predisposes offspring to renal fibrosis and that damage is worsened when offspring are exposed to an adverse environment in later life. Importantly, glomerulosclerosis and tubulointerstitial fibrosis are the final common pathways in a variety of kidney diseases leading to end-stage renal failure (ESRF) (Ma & Fogo 2003; Zeisberg & Neilson 2010). Given that maternal obesity is now a very common complication of pregnancy and that individuals exposed to maternal obesity are more likely to have a poor diet themselves in later life, these results have important implications for the current CKD epidemic.

6.5.6 Limitations and future directions

One limitation of this study was that blood pressure was measured at a single time in the day. Employing telemetry would allow continuous monitoring and might highlight differences in blood pressure and heart rate in offspring exposed to maternal obesity and/or offspring obesity throughout the day and night. In turn, this might suggest potential mechanisms that could be involved in altering blood pressure and heart rate as discussed in section 6.5.2.

An increase in glomerular area was found in offspring exposed to post-weaning obesity, but not in offspring exposed to maternal obesity. However both offspring exposed to maternal obesity and/or offspring obesity displayed glomerulosclerosis. It would be interesting to measure the Bowman's space relative to glomerular tuft area in 6 month offspring as a measure of glomerular atrophy/obsolescence. Ultimately, this could help to ascertain the mechanisms behind glomerulosclerosis and whether these mechanisms differ between offspring exposed to maternal obesity and offspring exposed to post-weaning obesity.

Using electron microscopy to assess finer details within the glomeruli of offspring exposed to obesogenic environments may also help to illuminate the mechanisms behind glomerulosclerosis in these animals. In particular, assessing podocyte effacement and basement membrane thickening would be beneficial since these features tend to precede albuminuria and glomerulosclerosis, particularly in diabetic nephropathy and obesity related glomerulopathy (Bassi et al. 2016; D'Agati et al. 2016).

Another limitation of the current study was that renal function, and therefore the presence of CKD, was not directly assessed. Going forward, it would be beneficial to assess the presence of albuminuria in offspring exposed to maternal obesity and/or offspring obesity. Albuminuria is a central feature of CKD and is thought to be one of the main factors causing tubulointerstitial fibrosis, which was observed in offspring exposed to maternal obesity and/or offspring obesity at 6 months of age. A decrease in GFR is another central feature of CKD. In the current study, cystatin C was measured in 6 month offspring as a marker of GFR and was found to be increased in obese offspring, indicating impaired GFR and an increased risk of cardiovascular disease. Although cystatin C is considered to be a superior indicator of GFR relative to other endogenous markers, it is still confounded by non-renal factors. It would be therefore be beneficial to utilise an exogenous marker of GFR such as inulin to accurately assess GFR. However, measuring inulin is expensive and timely (and would require amendment to our existing home office project licence). Furthermore, although cystatin C is confounded by non-renal factors, paradoxically these factors make it a better predictor of CVD than an accurate measure of GFR.

A folch assay did not show a significant effect of maternal diet on offspring lipid levels despite histological observations suggesting that offspring exposed to maternal obesity had ectopic renal lipid deposition. It's possible that the folch method, being a general quantitative assessment, was not sensitive enough to detect the changes in lipid deposition suggested by imaging. In the future, it would be useful to conduct oil red O staining on frozen kidney sections, firstly to confirm whether the vacuoles seen in the kidneys of offspring exposed to obesogenic environments are definitely composed of lipid, and secondly to quantify lipid deposition in specific areas within the kidney. Nevertheless, the folch assay together with histological observations suggested lipid deposition was increased within the kidneys of offspring exposed to an obesogenic environment, and *Lpl* mRNA was decreased by maternal and/or offspring obesity. These results highlight the importance of measuring the expression of other components involved in lipid metabolism and storage within the kidney. In particular, it would be interesting to see whether exposure to maternal obesity programs components involved in lipid handling within the kidneys of offspring. Ultimately, these investigations might highlight why these offspring are predisposed to ectopic lipid deposition and renal fibrosis.

Lastly, in the current study offspring were studied at 6 months of age which is still relatively young given that laboratory mice typically live for around 2 years. It would be interesting to assess renal health in even older offspring. In particular it would be important to determine whether the damage observed in offspring exposed to maternal and post-weaning obesity at 6 months leads to an impairment in renal function in older age, especially since the current results suggest that ageing is an important catalyst for renal damage in programmed offspring.

6.5.7 Conclusions

The results presented in this study demonstrate that a post-weaning obesogenic diet is sufficient to induce classic features of the metabolic syndrome in 6 month old offspring including obesity, an altered serum metabolic profile and increased blood pressure. Additionally, an offspring obesogenic diet led to changes in renal morphology and features of renal damage which are classic of obesity related renal disease. Importantly, maternal obesity exacerbated many of the adversities seen in offspring exposed to post-weaning obesity, the most striking of which was renal fibrosis. Since renal fibrosis is the final common feature of a number of renal diseases leading to ESRF, these results have important implications for the current CKD epidemic. Obesity prevalence has risen dramatically in recent years concurrently with the prevalence of CKD. These results suggest that maternal obesity could be contributing to the increase in chronic kidney diseases, and that interventions designed to target certain aspects of maternal obesity may aid in the prevention of CKD in future generations.

6.5.8 Summary

- At 6 months of age offspring exposed to a post-weaning obesogenic diet showed increases in weight, adiposity, serum insulin and cholesterol and a decrease in FFA. Exposure to maternal obesity accentuated increases in offspring weight, fat mass and cholesterol levels.
- Offspring exposed to a post-weaning obesogenic diet showed an increase in blood pressure at 6 months of age.
- Medulla and cortex areas and glomerular areas were increased, and glomerular density was decreased, in 6 month old offspring exposed to a post-weaning obesogenic diet.
- Cystatin C was increased in offspring exposed to a post-weaning obesogenic diet at 6 months of age, indicative of a decrease in GFR.
- Intra-renal lipid deposition was increased in offspring exposed to post-weaning obesity at 6 months of age. *Lpl* mRNA was decreased in offspring exposed to maternal obesity and/or offspring obesity indicative of impaired renal lipid metabolism.
- Gene expression relating to inflammation and fibrosis was altered in offspring exposed to maternal obesity and/or offspring obesity.
- Glomerulosclerosis and tubulointerstitial fibrosis were increased in offspring exposed to maternal obesity and were exacerbated by a post-weaning obesogenic diet.

Chapter 7. Maternal exercise in obese dams increases markers of renal morphogenesis in fetal offspring

7.1 Introduction

It is evident from the data presented so far in this thesis that exposure to maternal obesity alters offspring renal morphology in early life and has effects on offspring renal health in later life. As discussed in Chapter 3, there are a number of characteristics associated with maternal obesity that could be responsible for adverse outcomes in the offspring. It is therefore important to address the question of which maternal obesogenic factors could be involved in programming of the offspring kidney.

7.1.1 Maternal exercise has been shown to be beneficial for offspring health

7.1.1.1 Epidemiological studies

Exercising during pregnancy has been shown to be safe and beneficial for the mother's health regardless of BMI, improving overall maternal cardiovascular function and blood lipid regulation, and decreasing the risk for adverse complications during labour and delivery (May et al. 2016). Furthermore, meta-analyses have demonstrated that maternal exercise during human pregnancy is associated with a decreased risk of GDM (Russo et al. 2015; Sanabria-Martínez et al. 2015) and offspring of LGA (Wiebe et al. 2015), suggesting that exercise improves the maternal metabolism and potentially the associated outcomes in the offspring. Accordingly, fetal cardiovascular function has been shown to be improved in response to maternal exercise; at 39 weeks gestation offspring exposed to regular maternal exercise showed a reduced heart rate and increased heart rate variability compared with unexposed offspring (May et al. 2010), indicative of heathy autonomic regulation. Importantly, maternal exercise may induce long-lasting beneficial changes in the offspring. It was found that moderate maternal exercise during pregnancy reduced the risk of overweight/obesity in 8 year old children (Mourtakos et al. 2015). Together these studies highlight that exercise during pregnancy can have beneficial implications for metabolic and cardiovascular health in both mother and child.

7.1.1.2 Animal studies

Studies in animals have also shown positive results in offspring in response to maternal exercise. Female rats provided with a running wheel during gestation demonstrated improved glucose tolerance, decreased insulin levels, reduced hepatic glucose production and increased skeletal muscle glucose uptake following a hyperinsulinaemic–euglycaemic clamp, when compared to females housed in a standard cage during gestation (Carter et al. 2013). In mice, maternal exercise also reduced the percentage fat mass in exposed offspring in adulthood. Remarkably, mice exposed to maternal exercise were also protected from developing hepatic steatosis after being exposed to a high fat diet from weaning (Sheldon et al. 2016). Interestingly, it has been demonstrated that voluntary exercise is increased in adult offspring exposed to maternal exercise during pregnancy (Eclarinal et al. 2016). These studies emphasise the protective effects of maternal exercise on offspring health and support human findings. Importantly, these studies emphasise that the benefits of maternal exercise persist in offspring through to adulthood.

A few studies have now demonstrated that maternal exercise in obese mothers can be an effective intervention for improving offspring outcomes. In male mice, maternal exercise before and during pregnancy prevented the glucose intolerance, raised insulin levels and adiposity associated with a maternal high fat diet (Stanford et al. 2015). Likewise, exercise in high fat diet fed or obesogenic diet fed obese mouse dams improved glucose and insulin metabolism in male offspring (Raipuria et al. 2015; Fernandez-Twinn et al. 2017). Maternal exercise in high fat fed dams also prevented hyper-methylation of peroxisome proliferator-activated receptor γ co-activator-1α (*Pgc-1 α*) and increased *Pgc-1 α* expression in offspring skeletal muscle, indicative of improved mitochondrial biogenesis and oxidative metabolism (Laker et al. 2014). These changes were concomitant with improved age associated metabolic dysfunction. This study highlights that improved health in offspring due to maternal exercise might be partly due to epigenetic alterations.

From the evidence presented, it is clear that maternal exercise can positively influence both maternal and offspring metabolic health, and that exercise may be a useful intervention for preventing adverse offspring outcomes associated with maternal over-nutrition/obesity. This raises the possibility that maternal exercise might also be an effective intervention for preventing adverse renal outcomes in offspring exposed to maternal obesity.

7.1.2 Factors associated with maternal obesity have been shown to affect the offspring kidney.

As described in chapter 3, our diet-induced obesity model results in increased weight, adiposity and insulin levels and impairs glucose tolerance in obese dams during gestation when compared to control dams. Cholesterol levels may also be increased in obese dams during gestation or lactation since levels were observed to be increased at weaning (chapter 3). There is evidence that some of these maternal characteristics may affect the developing offspring kidney.

7.1.2.1 Glucose

There is abundant evidence from animal models that maternal hyperglycaemia is a potent factor affecting offspring renal development. Maternal diabetes induced by STZ injections at E6.5-8.5 in mice led to maternal hyperglycaemia by E12.5 (Hokke et al. 2013). Offspring of these diabetic dams showed growth restriction, a reduced ureteric tip number and ureteric tree length by E14.5, and a reduction in nephron number by E18.5. Insulin treatment at E13.5 normalised maternal glucose levels but did not rescue offspring nephron number, indicating that maintaining glucose homeostasis in early pregnancy, and thus ensuring adequate Ureteric Bud (UB) branching, is crucial for normal nephrogenesis in offspring. Conversely, embryonic mouse kidney explants cultured *ex vivo* showed increased paired box 2 (*Pax2*) (a master regulator of UB branching and nephrogenesis) gene expression and increased UB branching following high glucose exposure (Zhang et al. 2007). The authors found *in vivo* that high glucose (induced by maternal STZ administration at E13) decreased offspring body weight and kidney size. *Pax2* expression was increased in the UB and UB branching was increased, however nephrogenesis was unaffected (Zhang et al. 2007). It was suggested that this could be due to the fact that glucose also stimulates apoptosis within the UB and developing nephron, and that apoptosis may play an important role in the adverse effects in the kidney seen following early life exposure to diabetes/hyperglycaemia. In support of this, another study of maternal diabetes induced by STZ showed decreased offspring birth weight, kidney weight and nephron number. Importantly, an increase in apoptotic podocytes was observed along with active caspase 3 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation (Tran et al. 2008). In another study, offspring exposed to STZ-induced maternal diabetes had smaller kidneys, a decreased number of nephrons and showed evidence of apoptotic cells in collapsed nephrons and failed migration/invasion of the UB into the metanephric mesenchyme (Lin et al. 2013). These studies suggest that maternal hyperglycaemia could induce adverse offspring renal development via altered UB morphogenesis or apoptosis.

Nephron deficit is commonly observed with increased glomeruli areas or glomerular hypertrophy. These two factors can be inter-related since a reduction in nephron number would promote

hypertrophy and hyperfiltration in the remaining nephrons in order to maintain overall renal filtration rates. However, studies of maternal diabetes induced by STZ have shown that glomerular hypertrophy can occur in offspring independently of a reduction in nephron number (Rocha et al. 2005; Magaton et al. 2007). These studies highlight that maternal hyperglycaemia could be a factor promoting the increased glomeruli areas seen in offspring exposed to maternal obesity in this thesis.

7.1.2.2 Insulin

Insulin cannot cross the placenta. Therefore, maternal hyperinsulinaemia/insulin resistance can only affect the fetus by modifying other factors. Maternal hyperinsulinemia or insulin resistance could potentially affect the offspring kidney through increasing the availability of lipids. Insulin infusion in women with GDM does not decrease free fatty acids to the same degree as healthy women, suggesting that the availability of FFAs to the fetus is increased by insulin resistance (Catalano & Hauguel-De Mouzon 2011). In support of this hypothesis, maternal serum triglycerides have been shown to correlate with offspring abdominal circumference and fat mass at birth (Catalano & Hauguel-De Mouzon 2011).

In a model where dams were haplo-insufficient for IRS-1 making them insulin resistant and hyperinsulinaemic but not overweight or hyperglycaemic, exposed male mice showed impaired lipid metabolism, increased lipid deposition in the liver, as well as hyperglycaemia and hyperinsulinaemia (Isganaitis et al. 2014). The authors also found that maternal insulin resistance increased lipids within the maternal circulation and postulated that offspring could be exposed to increased lipids through hydrolysis of lipoproteins in the placenta and increased FFA transport to the fetus. This study suggests that maternal insulin independent of other factors can modify the offspring metabolism. In support of this, in the current thesis and in published findings from our laboratory, dams were hyperinsulinaemic during gestation and offspring showed hyperinsulinaemia at 8 weeks of age (see chapters 3 & 5) (Fernandez-Twinn et al. 2017)**.** Although there are no studies on the direct effect of insulin on the developing kidney, the evidence discussed suggests that maternal hyperinsulinaemia could affect renal development in offspring exposed to maternal obesity in the current study by altering the availability of other nutrients.

7.1.2.3 Lipids

Studies addressing the specific impact of increased lipid availability on offspring kidney development are lacking. However, one study showed that a maternal high fat diet which led to maternal hyperinsulinaemia, but did not affect maternal glucose tolerance or weight during late pregnancy, led to increased nephron numbers in exposed offspring (Hokke et al. 2016). This suggests that increased

offspring lipid availability via a maternal high fat diet coupled with hyperinsulinaemia, could enhance nephrogenesis.

7.1.2.3 Placental Insufficiency

Maternal obesity is associated with placental insufficiency and as such, obesity increases the risk for SGA infants (Rajasingam et al. 2009; McCowan et al. 2013). Our maternal diet-induced obesity model leads to placental insufficiency as evidenced by increased placental lipid deposition and hypoxia, and fetal growth restriction (Fernandez-Twinn et al. 2017). Models of maternal placental insufficiency induced by uterine artery ligation have shown that offspring are growth restricted and have fewer nephrons (Sanders et al. 2004; Wlodek et al. 2008), and an increased glomerular size (Sanders et al. 2004). The effects of placental insufficiency on the offspring kidney appear to be very similar to models of maternal under-nutrition and protein restriction (see chapter 1, section 1.5.1.), most likely due to the fact that all these models reduce nutrient availability to the fetus. Offspring exposed to maternal obesity in the current thesis showed growth restriction at birth and glomerular hypertrophy (see chapter 4 and 5), supporting a role for placental insufficiency in the programming of the offspring kidney by maternal obesity.

7.1.3. The maternal exercise model

Our laboratory has a model of maternal exercise intervention during pregnancy in obese dams. We have demonstrated that exercise improves glucose tolerance, and restores insulin levels to control levels, in obese dams during late pregnancy. This is independent of leptin levels, fat mass and body weight, which remain elevated in obese and exercised dams relative to control dams at E1 and E19 (Fernandez-Twinn et al. 2017). Table 7.1 also shows the fasting glucose levels and fed serum lipid levels in dams in late pregnancy in control, obese and obese exercised dams. HDL was increased in obese dams and rescued by exercise, however all other lipids and glucose were unchanged by exercise. This suggests that any changes in offspring exposed to maternal obesity with exercise as compared to offspring exposed to obesity alone are due to the correction of insulin levels and glucose tolerance.

Table 7.1. *Maternal serology at E18-E19.*

	Control (n=6)	Obese (n=9)	Ob-Ex (n=7)
Cholesterol (mmol/L)	1.32 ± 0.08	1.51 ± 0.10	1.39 ± 0.09
LDL (mmol/L)	0.43 ± 0.12	0.53 ± 0.04	0.49 ± 0.04
HDL (mmol/L)	0.38 ± 0.07	0.66 ± 0.06*	0.59 ± 0.06
Triglycerides (mmol/L)	1.12 ± 0.09	0.67 ± 0.04***	0.70 ± 0.04***
FFA (mmol/L)	0.60 ± 0.09	0.56 ± 0.03	0.43 ± 0.05
Fasting tail glucose (mmol/L)	8.1 ± 0.43	8.74 ± 0.36	8.59 ± 0.42

All lipids were measured from serum taken from fed dams at E19. Tail glucose was measured following a 4 hour fast at E18. N numbers are presented within the table. 1-way ANOVA; stars represent significance relative to control group following a post-hoc test, *p<0.05, **p<0.001, ***p<0.0001.

Additionally, a rescue of placental function could improve outcomes in offspring exposed to maternal obesity with exercise as opposed to offspring exposed to obesity alone. Placental weights were shown to be similar between control, obese and obese exercised dams. Despite this, obese dams showed indices of placental insufficiency including increased placental lipid deposition and evidence of hypoxia (increased Hypoxia-inducible factor 1-alpha (HIF1A) protein levels), which was rescued by exercise (Fernandez-Twinn et al. 2017). Furthermore, maternal insulin levels correlated significantly with placental HIF1A protein levels, suggesting that hyperinsulinaemia may promote placental hypoxia. The current working hypothesis in our laboratory is that maternal hyperinsulinemia/insulin resistance may negatively affect the amount of available nitric oxide, thereby impairing uteroplacental vasodilation, leading to decreased placental perfusion and ultimately resulting in placental-fetal hypoxia. Despite the improvement in placental health, maternal exercise did not rescue the growth restriction seen in fetuses exposed to maternal obesity (Figure 7.1). However, at 8 weeks of age, male offspring of exercised obese dams had normalised fasting insulin levels and IRS-1 protein levels within epididymal fat suggesting an improvement in insulin sensitivity due to maternal exercise. The above evidence suggests that in our model of maternal obesity, maternal hyperinsulinaemia may be a key programming factor mediating effects on the offspring, and that maternal exercise therefore might

be an effective intervention to prevent adverse outcomes in offspring. Renal outcomes associated with maternal insulin, glucose and placental insufficiency, as discussed above, may therefore be ameliorated by maternal exercise. Thus, the aim of the current study was to compare the impact of maternal obesity, with or without exercise, on markers of renal development in E19 fetuses. Chapter 1, section describes some of the key genes involved in renal development. Table 7.2 details the genes assessed in this chapter including a rationale for their evaluation. The offspring used for renal studies in the current study were generated from the same cohort of dams described above (Fernandez-Twinn et al. 2017).

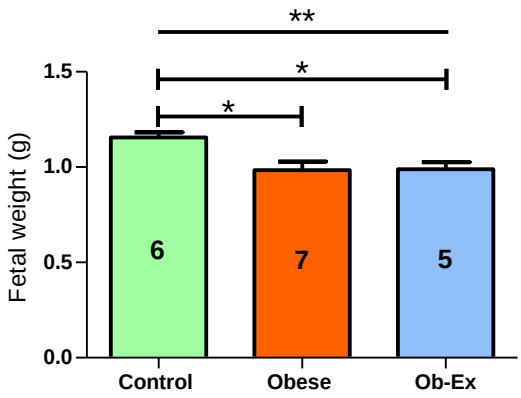

Figure 7.1. **E19 fetal weights**. N numbers are presented within the graphs. 1-way ANOVA: Uncapped lines indicate an overall significant effect of maternal environment. Capped lines denote significance between groups following a post-hoc test. *p< 0.05, **p< 0.001.

Table 7.2. *Genes selected for this study and their role in renal development.*

Gene	Role in renal development
Gdnf	Stimulates growth and branching of the UB (an important precursor to nephrogenesis) through receptors RET and GFRA1 (Fisher et al. 2001). Heterozygous mice have fewer nephrons (Cullen-McEwan et al. 2003).
Ret	Downstream of GDNF and so promotes branching of the UB (Fisher et al. 2001).
Gfra1	Downstream of GDNF and so promotes branching of the UB (Fisher et al. 2001).
Wt1	Master regulator of nephrogenesis. Expressed in induced metanephric mesenchyme and in cells undergoing glomerular specialisation (Abrahamson & Steenhard, 2008).
Pax2	Upstream of GDNF and so important for UB branching (Reidy & Rosenblum, 2009). Heterozygous mice exhibit increased apoptosis and decreased UB branching leading to fewer nephrons (Abrahamson & Steenhard, 2008).
Ren1	Upsteam of Angiotensin II, AGTR2 and AGTR1. Renin producing cells originate from mesenchyme at E11 in mice (when ureteric bud branching is just beginning) (Yosypiv 2012).
Agtr2	Inhibits growth and proliferation in developing kidney. Mutant mice have decreased apoptosis in mesenchymal cells surrounding ureteric bud (Yosypiv 2012).
Agrt1	Stimulates ureteric bud branching and growth (Yosypiv 2012).
Bax	Pro-apoptotic. Pax2 decreases expression of *Bax* therefore favouring growth rather than apoptosis. Bax upregulated in rat IUGR kidneys (Pham et al. 2003).
Casp3	Pro-apoptotic. Been demonstrated to be increased within the renal tubules of offspring exposed to maternal diabetes (Tran et al. 2008).

Gdnf (Glial derived neurotrophic factor), *Ret* (Receptor tyrosine-protein kinase), *Gfra1* (Glial derived neurotrophic factor receptor alpha), *Wt1* (Wilms tumour 1), *Pax2* (Paired box 2), *Ren1* (Renin), *Agtr2* (Angiotensin II receptor type 2), *Agtr1* (Angiotensin II receptor type 1), *Bax* (BCL2 associated X, Apoptosis regulator), *Casp3* (Caspase 3).

7.2 Aims

The aim of this chapter was:

- To assess markers of renal development in E19 fetuses exposed to maternal obesity with or without exercise.

7.3 Methods

7.3.1 Generation of the maternal exercise model

From weaning, females were either fed a standard chow or a highly palatable obesogenic diet as described in chapter 2, section 2.1. Females were mated for a first pregnancy (P1) as described in chapter 2, section 2.1. Females were maintained throughout on their respective diets for approximately 6 weeks until the body mass of "obesogenic" assigned females comprised at least 10g fat mass as measured by TD-NMR (minispec TD-NMR, Bruker Optics, MA). Control dams had less than 5g total fat mass. Some obese dams were then randomly assigned for exercise. Exercise was conducted in the dark under red light and was timed around the beginning of the dark "active" cycle for the mice. 1 week prior to mating for a second pregnancy, dams assigned to the exercise protocol were trained to run on a treadmill for 20 minutes per day, starting at 5 m/min on the first day and with daily incremental increases in speed until they were running at a final speed of 12.5 m/min on day 5. Completion of the training week indicated dams were competent runners. Competent runners were then rested for 2 days and mated for a second pregnancy (P2), which was confirmed by the presence of a copulatory plug. Exercise was maintained at the final running speed for 5 consecutive days a week and until gestational day 17 (E17). Aged matched control and obese dams were mated and maintained on their respective diets throughout gestation as described in chapter 2, section 2.1. On day 18 of pregnancy, dams were fasted for 4 hours before undergoing a glucose tolerance test. Dams were then re-fed overnight before being killed the next day and the fetuses dissected on E19. Dams in all groups were weighed weekly and TD-NMR measurements were conducted on day 1 and day 19 of pregnancy. The protocol described resulted in the generation of E19 fetuses from three experimental groups (Control, Obese and Ob-Ex) as described in Figure 7.2.

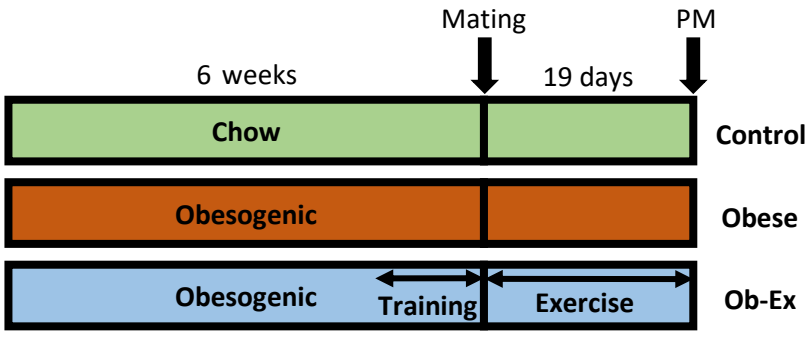

Figure 7.2. **Schematic diagram detailing the exercise study protocol.**
Control = fetuses exposed to a maternal chow diet from conception to E19
Obese = fetuses exposed to a maternal obesogenic diet from conception to E19
Ob-Ex = fetuses exposed to a maternal obesogenic diet with maternal exercise from conception to E19.

7.3.2 Fetal renal mRNA expression.

Following dissection, fetal kidneys from each litter were pooled and stored at -80^{o}C for later analysis. RNA extraction, reverse transcription and qPCR were performed as described in chapter 2, section 2.6. All primers were exon-exon spanning to avoid the amplification of genomic DNA. Primers were designed to detect the following genes; *Gdnf* (Glial derived neurotrophic factor), *Ret* (RET receptor tyrosine kinase), *Gfra1* (Glial derived neurotrophic factor receptor alpha), *Wt1* (Wilms tumour 1), *Pax2* (Paired box 2), *Ren1* (Renin 1), *Agtr1a* (Angiotensin II receptor type 1), *Agtr2* (Angiotensin II receptor type 2), *Casp3* (Caspase 3), *Bax* (BCL2 associated X, Apoptosis regulator). The expression level of these genes of interest were normalised to the geomean of the housekeeping genes (*Hprt, Gapdh* and *Sdha*) (the expression of which did not change between groups), as described in chapter 2, section 2.6.10. Table 7.3 shows the primers and their sequences used for E19renal gene expression detection.

Table 7.3. *Primer sequences for E19 renal genes.*

Gene name	**Forward primer (5'→3')**	**Reverse primer (3'→5')**
Gdnf	GATTCGGGCCACTTGGAGTT	TTCAGGCATATTGGCGGCG
Ret	AGAGCAGAGACTACTTGGACCT	AGTAAATGCATGTGAAATTCTACCA
Gfra1	GTCTCTCTACAACTGCCGCT	CTTTGGAAATGTGTTCCACTGA
Wt1	TCCGAGGCATTCAGGATGTG	TGCTTCCGGCTATGCATCTG
Pax2	CGTTGTGACCGGTCGTGATA	TGCTGAATCTCCAAGCCTCA
Ren1	GCACCGCTACCTTTGAACGA	TCGCCGTAGTACTGGGTATTC
Agtr1a	AGTTGGGAGGGACTGGATGA	GTTAAGTCCGGGAGAGCAGC
Agtr2	TTTTAAGGAGTGCATGCGGG	AAAAGGACGGCTGCTGGTAA
Casp3	GCTTGGAACGGTACGCTAAG	CCACTGACTTGCTCCCATGT
Bax	AAACTGGTGCTCAAGGCCC	CTTGGATCCAGACAAGCAGC
Hprt	ACAGGCCAGACTTTGTTGG	ACTTGCGCTCATCTTAGGT
Gapdh	CAACGGGAAGCCCATCAC	GCCTCACCCCATTTGATGTT
Sdha	TTACAAAGTGCGGGTCGATGA	TGTTCCCCAAACGGCTTCTT

7.3.3 Fetal renal protein expression

7.3.3.1 Homogenisation of kidney tissue

Frozen kidneys were powdered on dry ice and 30mg of powdered tissue was weighed into a fresh tube before homogenising using a motorised tissue rupture (Qiagen, Sussex, UK) in ice-cold TK lysis buffer (0.3ml TK lysis for 30mg of sample) (see Table 7.4). Homogenised lysates were centrifuged at 13000rpm for 5 minutes at 4°C (Eppendorff centrifuge 5424, Hamburg, Germany). Supernatant was transferred into a clean eppendorf and stored at -80°C. The pellet containing cell debris and nuclei was discarded.

Table 7.4. *Buffers used for the homogenisation of kidney tissue in preparation for protein detection.*

Buffer	**Contents**
TK lysis buffer • Protease inhibitor cocktail was added immediately prior to use	50mM HEPES (pH 8.0) 150mM Sodium Chloride 30mM Sodium fluoride 1mM Sodium orthovanadate 10mM Sodium pyrophosphate 10mM EDTA 1% Triton X-100 Protease inhibitor cocktail III (Calbiochem Novabiochem Biosciences, Nottingham, UK)
5x SDS buffer	25% (v/v) 1M TRIS (pH 6.8) 7.7% (w/v) Dithiothreitol 10% (w/v) Sodium dodecyl sulphate 50% (v/v) Glycerol Bromophenol blue dH_20

7.3.3.2 Measuring protein concentration

Protein concentration was determined for each of the samples using a Bicinchoninic acid assay. Stock bovine serum albumin (BSA) was serially diluted with dH_20 to create 6 standards (Sigma Aldrich, Poole, UK): S1: 1mg/ml, S2: 0.5mg/ml, S3: 0.25mg/ml, S4: 0.125mg/ml, S5: 0.0625mg/ml and S6: 0.03125mg/ml.

Kidney lysates were diluted in dH_20 (1:20 and 1:40 dilutions). A 96-well micro titre plate (Corning Inc., Corning, NY, USA) was loaded with 50µl sample dilutions and standards. A 50:1 dilution of bicinchoninic acid and copper (Cu2+) sulphate solution was made and 200µl was added to each of the wells. The plate was then incubated for 5 minutes at room temperature. During this time, cuprous ions (Cu1+) were generated producing a purple colour that was proportional to the overall protein concentration. Absorbances were analysed by the ASYS expert plus micro plate reader (Biochrom, Cambridge, UK) at 595nm. A standard curve was generated using the BSA standards (Figure 7.3). Samples were then interpolated onto the standard curve to calculate the protein concentration of kidney lysates.

25µl 5x sodium dodecyl sulfate (SDS) buffer (Table 7.4) was added to 100µl kidney sample lysate. All lysates were standardised to 1mg/ml by addition of 1xSDS (5xSDS 1 in 5 dilution). To prevent protein degradation as a result of freeze thawing, lysates were aliquoted into smaller volumes (20µl) and stored at -80°C.

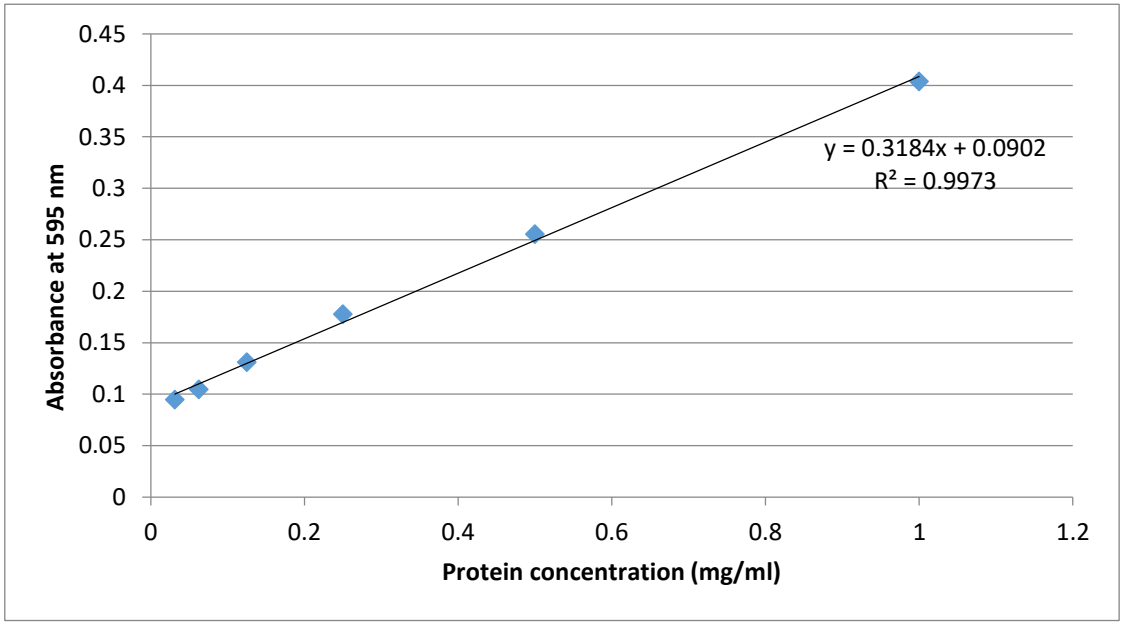

Figure 7.3 **BSA standard curve used to interpolate the protein concentration of kidney samples.**

7.3.3.3 SDS-PAGE

Using discontinuous polyacrylamide gel electrophoresis, kidney samples (15µl) were separated via SDS polyacrylamide gel electrophoresis (SDS-PAGE). Resolving gels were used with 12% acrylamide. Stacking gels contained 5% acrylamide (Table 7.5). Gels were set between glass plates held in place by a casting stand (Bio-Rad, Hertfordshire, UK). After gels were set, they were placed in an electrophoresis tank (Bio-Rad mini protean 3, Bio-Rad, Hertfordshire, UK) with running buffer (Table 7.5). Samples were heated to 95°C before SDS-PAGE. A maximum of 14 samples could be loaded onto

a gel at any one time. A pre-stained protein ladder from 10-250kDa (Thermo Fisher Scientific, Waltham, USA) was also loaded onto the gel. Gels were run at a constant voltage (150V) for 1.15hrs and electrophoresis was terminated when the gel front reached the bottom of the gel.

Table 7.5. *Gels and buffers used for SDS-Page in preparation for protein detection.*

Gel/buffer	**Contents**
Resolving gel	12% Acrylamide (40%) 0.05% Bisacrylamide (2%) 37.5mM Tris-HCL 1% (w/v) 20% SDS 0.05% TEMED 0.0005% APS (10%)
Stacking gel	5% Acrylamide (40%) 0.05% Bisacrylamide (2%) 12.5mM Tris-HCL 1% (w/v) 20% SDS 0.05% TEMED 0.0005% APS (10%)
Running buffer	25mM TRIS-base 200nM Glycine 0.1% SDS

7.3.3.4 Coomassie blue staining

The first gel of the experiment was stained with Coomassie brilliant blue R-250 stain (Bio-Rad, Hertfordshire, UK) for 1 hour at room temperature. The gel was then rocked in Coomassie brilliant blue R-250 de-staining solution until the lanes and protein bands were clearly visible. An image was taken by scanning the gel and staining intensities for each lane were measured. Equal intensities of the protein bands in each lane indicated that the samples were loaded evenly and that each sample contained the same amount of protein overall. This insured that any differences in specific proteins between samples following western blotting would be due to a true biological variability. Figure 7.4 shows a comassie stained gel from E19 samples.

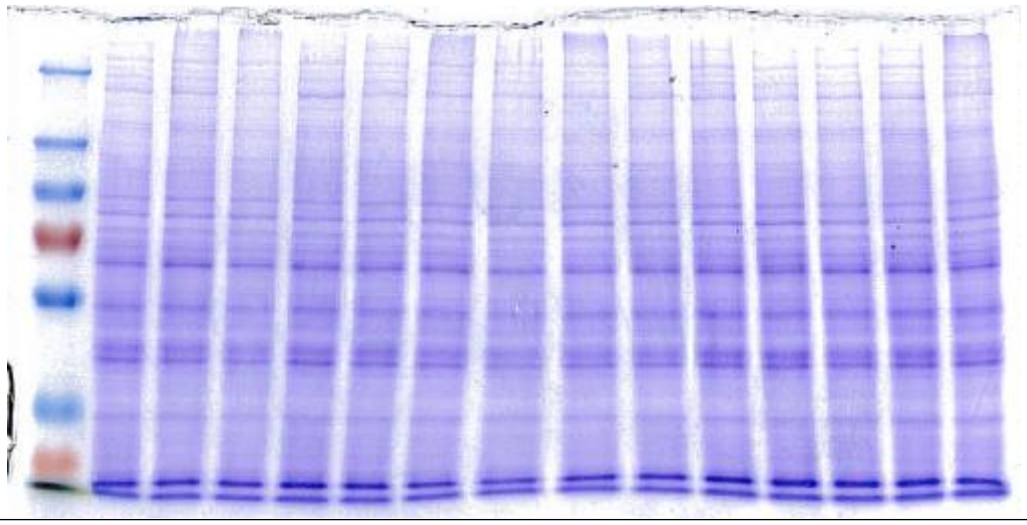

Figure 7.4 **Coomassie blue staining of E19 kidney protein samples.**

7.3.3.5 Western blotting

Proteins separated by SDS-PAGE were transferred to a Polyvinylidene difluoride (PVDF) Immobilon-P membrane (Millipore, Billerica, MA, USA) using semi-dry transfer. Prior to transfer, the membrane was placed in 100% methanol, dH_20 and then 1x transfer buffer (Table 7.6) each for roughly a minute. Three pieces of filter paper the same size as the gel were soaked in 1x transfer buffer and layered onto the transfer apparatus, followed by the PVDF membrane, the resolving gel containing the separated proteins and a further 3 pieces of filter paper. Air bubbles were removed by rolling with a clean pipette. Semi-dry transfer was performed at room temperature for 105 minutes at a constant current of 200mA using a Bio-Rad transfer blot electrophoretic transfer pack (Bio-Rad, Hertfordshire, UK). After transfer, PVDF membranes were washed briefly to remove any adhering acrylamide and placed in 5% non-fat dry milk (NFDM) blocking buffer (see Table 7.6) for 1 hour at room temperature.

After blocking, the membrane was washed three times with Tris-buffered saline with Tween 20 (TBS/T) (Table 7.6) for 5 minutes each and was then incubated with the primary antibody overnight at 4^{o}C. The primary antibodies (phosphorylated 44/42 mitogen activated protein kinase [ERK1/2] 9102S and phosphorylated p38 mitogen activated protein kinase [p38 MAPK] 9211S [Cell signalling]) were diluted (1 in 1000) in 1x TBS/T with 5% BSA in accordance with the antibody datasheets. After an overnight incubation, the membrane was washed three times for 5 minutes with TBS/T. The membrane was subsequently incubated with horseradish peroxidise-conjugated affinity rabbit secondary antibody (Jackson ImmunoResearch, Stratech, Newmarket, UK). The secondary antibody was diluted (1 in 1000) in 5% NFDM (Table 7.6). A further three washes for 5 minutes in TBS/T were carried out to minimise non-specific antibody binding.

Table 7.6. *Buffers used for western blotting in preparation for protein detection.*

Buffer	**Contents**
Tris-buffered saline (TBS) (10x, pH 7.6)	50nM Tris-base 150mM Sodium Chloride
1x transfer buffer	50mM Tris-base 40mM Glycine 0.15% (w/v) SDS 20% (v/v) Methanol
TBS/T	1x TBS 0.1% Tween-20
5% NFDM blocking buffer	5% (w/v) non-fat dehydrated milk TBS/T

7.3.3.6 Protein detection

After antibody incubation, protein bands were detected by chemiluminescence. Membranes were rocked in super signal west pico chemiluminescent substrate (GE Healthcare Life Sciences; Buckinghamshire, UK) for 5 minutes. Membranes were then imaged using Bio-Rad's Chemi-doc MP system (Bio-Rad Laboratories, Hertfordshire, UK).

7.3.3.7 Densitometry

Images taken with the charge coupled device camera included in the Bio-Rads Chemi-doc MP system were analysed using Image Lab software (Bio-Rad Laboratories, Hertfordshire, UK) using spot-densitometry.

7.3.3.8 Ponceau staining

Immediately after imaging for protein detection, the membranes were washed in TBS/T before rocking in ponceau stain for a few minutes, staining all proteins on the membrane pink. Visualising all the proteins on the membrane ensured that the transfer of proteins from the gel was even across the whole membrane (see Figure 7.5).

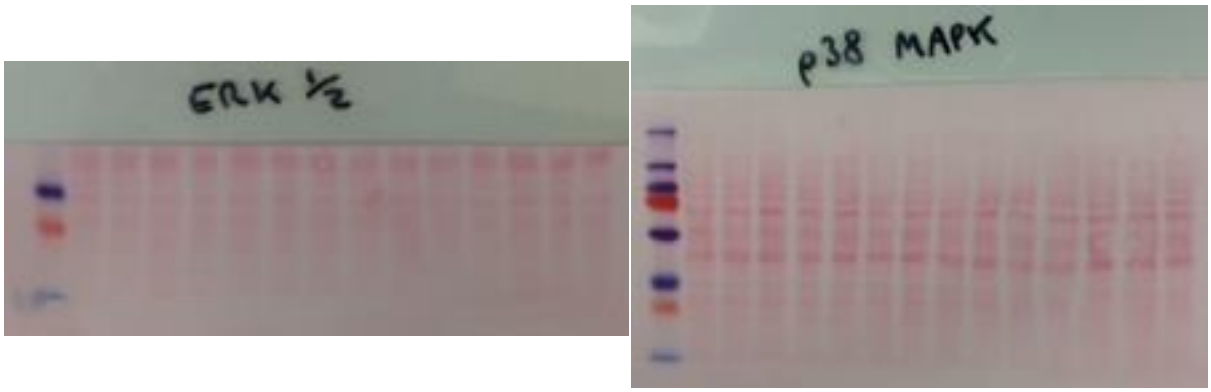

Figure 7.5. **Ponceau staining of E19 kidney proteins on PVDF membranes following transfer.**

7.3.4 Statistics

All statistical analyses were conducted using Prism 5.0 (GraphPad, CA, USA). All data were checked for normality by a Kolmogorov–Smirnov test. Statistical comparisons were performed using 1-way ANOVA. For all data sets, the mean and SEM are presented and $p<0.05$ is considered statistically significant. Sample sizes (n) are reported within each figure. For offspring data sets n represents the number of independent litters per experimental group.

7.4 Results

At E19, offspring renal *Gdnf* mRNA expression was significantly affected by the maternal environment, with a tendency for levels to be decreased in offspring exposed to maternal obesity and rescued by maternal exercise (Figure 7.6.A). Notably, offspring exposed to maternal obesity with exercise showed significantly increased levels relative to those exposed to maternal obesity alone (Figure 7.6.A). There was a borderline significant effect of the maternal environment on fetal *Ret* mRNA levels (p=0.06, Figure 7.6.B). *Gfra1* mRNA levels were significantly affected by the maternal environment with levels increased by the exercise intervention (Figure 7.6.C).

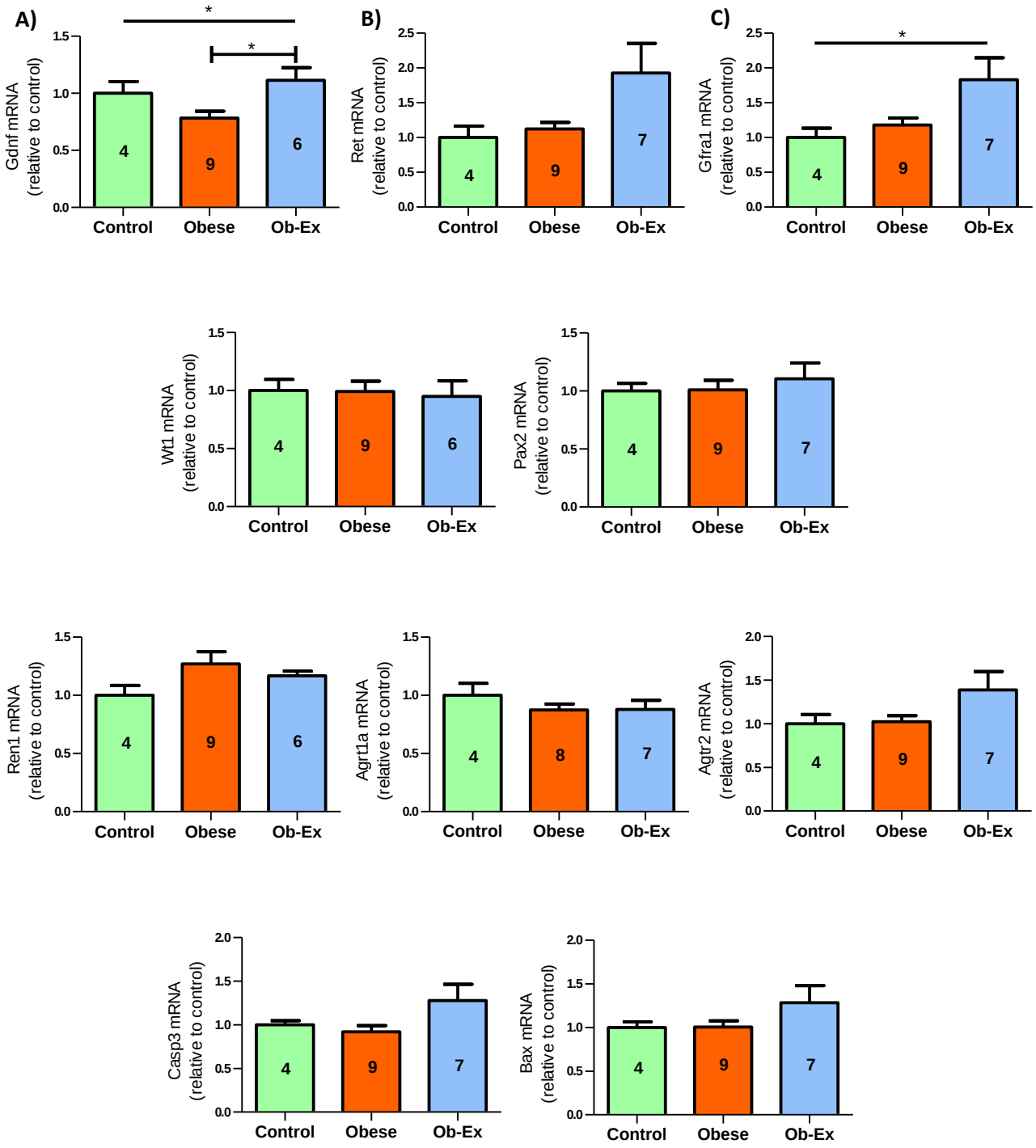

Figure 7.6. **Expression of genes important for ureteric branching in E19 fetal kidneys. A)** Glial derived neurotrophic factor **B)** RET receptor tyrosine kinase **C)** Glial cell line-derived neurotrophic factor receptor alpha **D)** Wilms tumour 1 **E)** Paired box 2 **F)** Renin 1 **G)** Angiotensin II receptor type 1 **H)** Angiotensin II receptor type 2 **I)** Caspase 3 **J)** BCL2 associated X, Apoptosis regulator. N numbers are presented within the graphs. 1-way ANOVA: Uncapped lines indicate an overall significant effect of maternal environment. Capped lines denote significance between groups following a post-hoc test. *p< 0.05.

There was a significant negative correlation between fetal renal *Gdnf* mRNA levels and maternal serum insulin levels at E19 (Figure 7.7.A). Conversely, *Ret* (Figure 7.7.B) and *Gfra1* (Figure 7.7.C) mRNA levels correlated positively with maternal serum LDL levels.

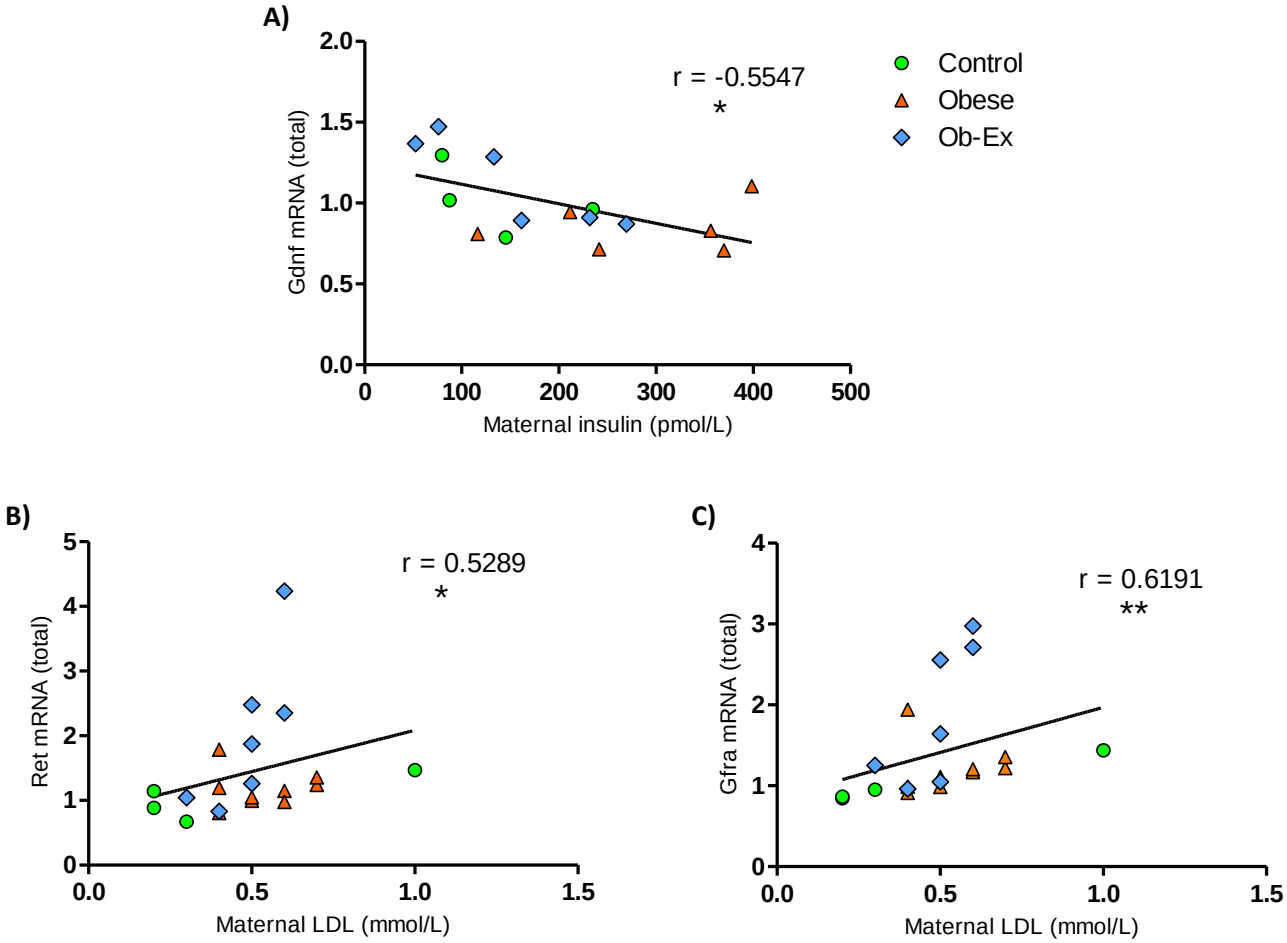

Figure 7.7. **Fetal ureteric branching gene expression correlated with maternal serum measures. A)** Maternal insulin and offspring Glial derived neurotrophic factor (Pearson's correlation, n = 4 control, 6 obese, 6 ob-ex) **B)** Maternal LDL and offspring RET Receptor Tyrosine Kinase (Spearman correlation, n = 4 control, 9 obese, 7 ob-ex) **C)** Maternal LDL and offspring Glial Cell Line-Derived Neurotrophic Factor Receptor Alpha (Spearman correlation, n = 4 control, 9 obese, 7 ob-ex). *p< 0.05, **p< 0.001.

At E19, p38 MAPK and ERK1/2 fetal renal protein levels were significantly affected by the maternal gestational environment (Figures 7.8.A & B). Offspring exposed to obese exercised mothers showed significantly higher levels when compared to offspring from both control and obese mothers (Figures 7.8.A & B).

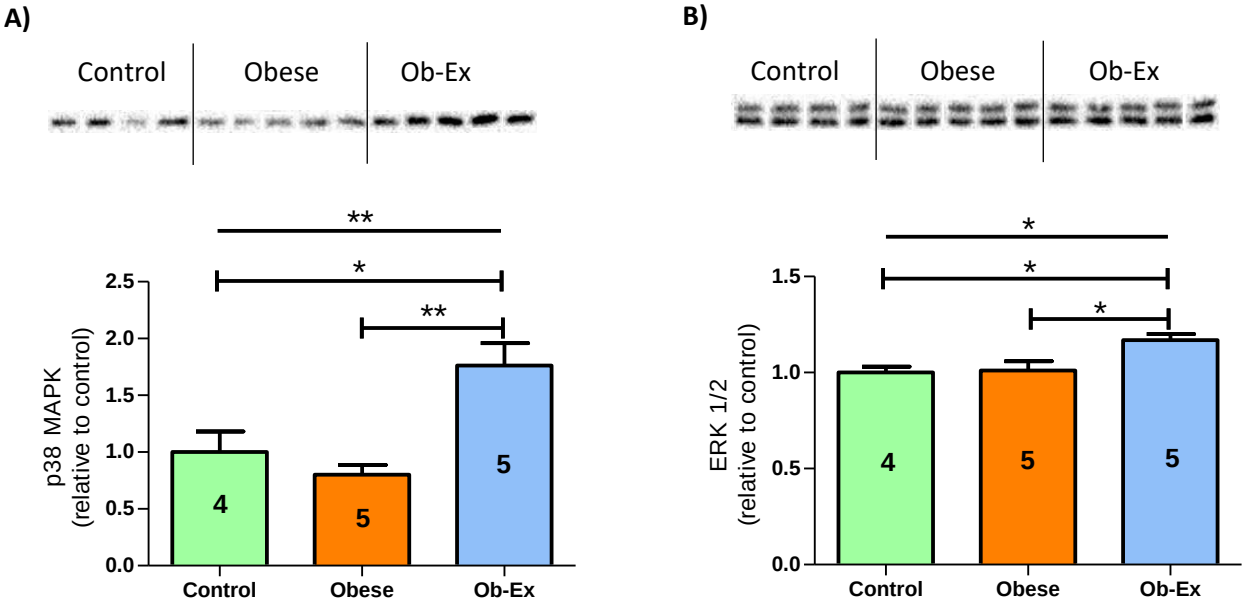

Figure 7.8. **Expression of proteins important for ureteric branching in E19 fetal kidneys. A)** Phosphorylated p38 mitogen activated protein kinase **B)** Phosphorylated p44/42 mitogen activated protein kinase. N numbers are presented within the graphs. 1-way ANOVA: Uncapped lines indicate an overall significant effect of maternal environment. Capped lines denote significance between groups following a post-hoc test. *p< 0.05. **p<0.001.

7.5 Discussion

The aim of this chapter was to compare markers indicative of renal development in fetal offspring exposed to maternal obesity with or without exercise throughout gestation.

7.5.1 Fetal renal development

Gdnf renal mRNA expression, which tended to be reduced in fetuses exposed to obesity alone, was increased to control levels in fetuses exposed to maternal obesity with exercise. *Gfra1* and *Ret* mRNA expression levels were also increased in fetuses exposed to maternal obesity with exercise. GDNF is secreted by the metanephric mesenchyme and binds to GFRA1 and its co-receptor RET on the UB in order to stimulate UB branching (Fisher et al. 2001) (see Figure 7.9). That *Gdnf* is important for bud branching is supported by observations that *Gdnf* null mice fail to form buds at all (Costantini 2010), GDNF supplementation in culture leads both to more UB branching and more nephrons, and heterozygous *Gdnf* mice have 30% fewer nephrons (Cullen-McEwen et al. 2003). These results suggest that offspring exposed to maternal obesity with exercise might have enhanced UB branching leading to increased nephrogenesis. This is supported by the observation that ERK1/2 protein expression (required for UB branching via GFRA1/RET (Fisher et al. 2001)) was increased in fetuses exposed to maternal obesity with exercise. Cells in the branch compete for their place in the tip domain, the higher level of GFRA1/RET signalling they have, the more likely they are to form part of the tip domain (Costantini 2010). Therefore, higher levels of *Gdnf, Gfra1* and *Ret* mRNA leading to the activated intracellular pathway in the kidneys of fetuses exposed to maternal exercise is indicative of rapid branching or spatially extensive branching. Furthermore, p38 MAPK has been shown to be important for branching morphogenesis via stimulation by bone morphogenetic protein 7 (Leung-hagesteijn et al. 2005; Smeeton et al. 2015) and was also increased in fetuses exposed to maternal obesity with exercise in the current study. Interestingly, there is evidence that p38 MAPK within the UB leads to *wnt11* gene expression which contributes to the maintenance of *Gdnf* expression in the metanephric mesenchyme (see Figure 7.9). Together the results discussed above suggest that maternal obesity with exercise might enhance fetal UB branching, an important precursor for nephrogenesis.

Alternatively, increased *Gdnf* in obese exercised dams could reflect increased apoptosis of developing renal cells. Increased GDNF signalling was shown to increase apoptosis and decrease cell migration in human proximal tubular cells, and to impair UB invasion into the metanephric mesenchyme and enhance apoptosis leading to the collapse of developing nephrons *in vivo* (Lin et al. 2013). There is therefore evidence that GDNF signalling can promote both UB branching and apoptosis. This isn't surprising given that optimal UB morphogenesis requires a delicate balance between cell proliferation

and apoptosis, which if tipped may promote abnormal renal development (Steer et al. 2004). The prevailing environmental conditions might dictate the balance between apoptosis and proliferation in the developing kidney via GDNF. In the aforementioned study, the increase in GDNF leading to failed cell migration and increased renal apoptosis was due to high glucose levels, and the authors showed that in the absence of glucose, GDNF increased cell migration *in vitro* (Lin et al. 2013). In the current study, obese dams showed impaired glucose tolerance and exposed offspring had decreased *Gdnf* (although not significant), and obese exercised dams showed optimal glucose tolerance and exposed offspring had increased *Gdnf*. Additionally maternal insulin, which would be expected to increase in response to maternal hyperglycaemia (a factor promoting excess glucose transport the fetus), correlated negatively with *Gdnf* expression levels. This suggests that glucose was not a factor promoting *Gdnf* expression in the current study and it is therefore unlikely that the increase in *Gdnf* in fetuses exposed to obese dams with exercise would enhance apoptosis.

Interestingly, there was a positive correlation between fetal renal *Gfra1* and *Ret* mRNA expression and maternal serum LDL levels, suggesting that LDL may enhance UB branching and nephrogenesis. As mentioned in section 7.1.2.3, a recent study showed that a maternal high fat diet slightly increased the ureteric tree volume in E18.5 offspring and increased the number of nephrons in E18.5 and PN21 offspring (Hokke et al. 2016). Importantly, dams on the high fat diet in that study showed normal glucose tolerance in late pregnancy. This highlights that an abundance of lipid availability to the fetus, in the absence of high glucose levels might enhance UB branching and nephrogenesis. It's possible that fetuses exposed to maternal obesity with exercise in the current study had a higher availability of fats compared to control fetuses due to the obesogenic diet, but were exposed to lower glucose levels than fetuses exposed to an obesogenic diet alone, due to the restoration of insulin levels and glucose tolerance in obese exercised dams. This provides a possible explanation for the increased *Gfra1/Ret* expression and downstream pathway in fetuses exposed to maternal obesity with exercise and highlights that these offspring may have enhanced nephrogenesis.

Linking to the point above, offspring exposed to maternal obesity with exercise may have showed indicators of enhanced UB branching because of the normalisation of maternal insulin levels leading to the restoration of placental function. This would be consistent with the notion that the availability of certain nutrients such as lipids might be enhanced in these offspring. In support of this, rats exposed to 50% maternal nutrient restriction during gestation had impaired UB morphogenesis which resulted in a nephron deficit (Awazu & Hida 2015). Therefore, in the current study offspring exposed to maternal obesity could be at risk of reduced UB branching due to placental insufficiency reducing overall nutrient availability. The observation that maternal exercise improves placental health in obese dams, together with the increased markers of UB morphogenesis in offspring of obese,

exercised dams suggests that placental viability might be important for renal development in our model.

Together the observations in fetal kidneys of offspring in the current study suggest that maternal obesity with exercise might enhance UB branching which is important for nephrogenesis. Of course, in the future it will be integral to measure the extent of branching and to count nephrons in these offspring in order to confirm this hypothesis.

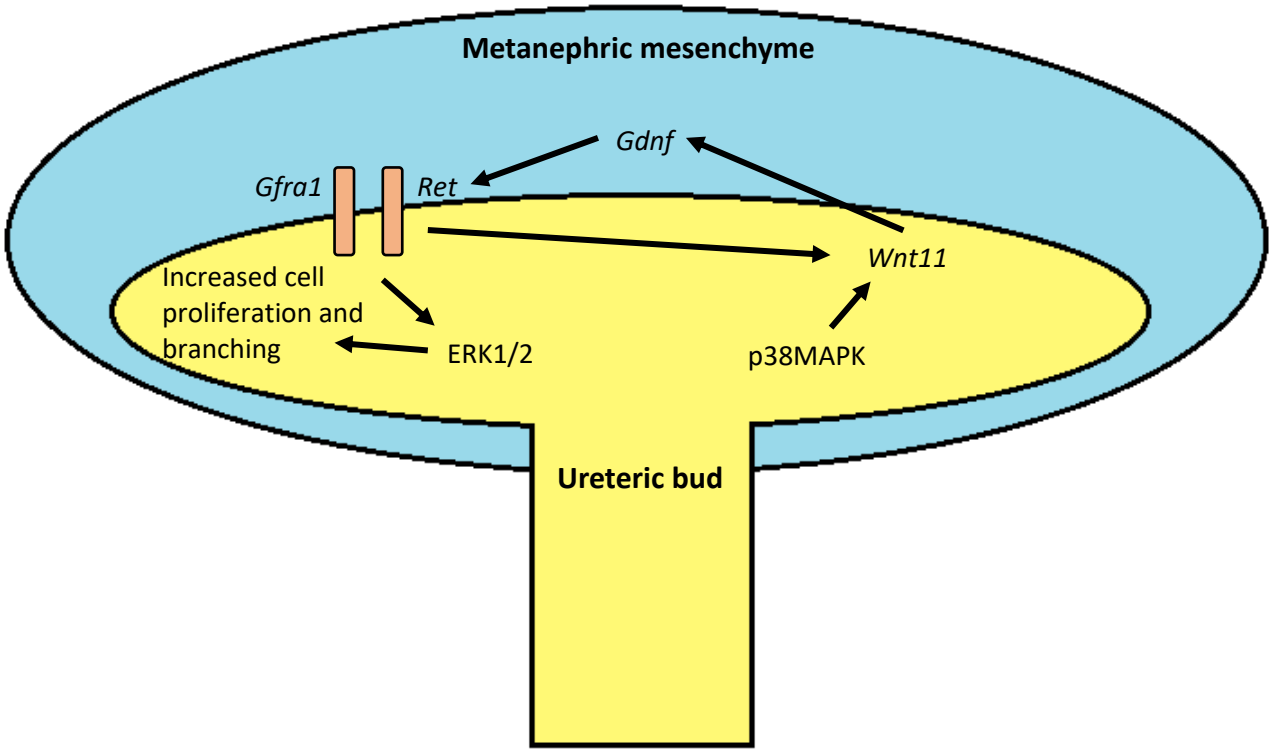

Figure 7.9. **Proposed pathway by which ureteric bud branching could be enhanced in offspring exposed to maternal obesity with exercise.** *Gdnf*, *Ret* and *Gfra1* mRNA expression and p38MAPK and ERK1/2 protein expression were increased in offspring exposed to maternal obesity with exercise. Pathway information was obtained from (Fisher et al. 2001) and (Smeeton et al. 2015).

7.5.2 Limitations and future directions

Maternal exercise in obese dams did not normalise the E19 fetal weights, despite the fact that indices of placental health were improved by maternal exercise, and adult offspring exposed to exercise showed improved insulin levels (Fernandez-Twinn et al. 2017) and cardiovascular function (unpublished). Our laboratory has hypothesised that maternal obesity leads to placental hypoxia which may impair oxygen and nutrient delivery to the fetus. Since maternal exercise in obese dams decreased a marker of placental hypoxia but offspring remained growth restricted in the current study, this hypothesis requires further investigation. Fetal hypoxia is known to negatively correlate with the mean velocity of blood flow in the fetal aorta (Soothill et al. 1986). Additionally, assessment of uterine, umbilical and middle cerebral aortic flow can be used to measure placental insufficiency (Krishna & Bhalerao 2011). In particular an abnormal ratio between uterine blood flow and fetal middle cerebral blood flow has been associated with lower gestational age of delivery, IUGR and greater risk of perinatal death in humans (Shahinaj et al. 2010). Importantly, IUGR with normal umbilical blood flow has been shown to be benign (Burke et al. 1990), highlighting that IUGR is not a certain indicator that the fetus is in poor condition. Measuring placental and fetal blood flow by doppler echocardiography would therefore allow a thorough assessment of placental function and fetal heath in our model. Placental health could also be assessed by radiolabelling certain nutrients and tracking their transfer from the maternal to the fetal circulation under conditions of *ex vivo* isolated placental perfusion (Cleal et al. 2007). These experiments would allow us to ascertain to what extent maternal obesity impairs placental function and whether maternal exercise is sufficient to fully restore placental function. These studies will be important if we are to discern the factors that are important for renal programming in the setting of maternal obesity.

The results presented in this chapter showed that E19 offspring exposed to maternal obesity with exercise demonstrated an increase in components in the pathway important for UB branching. This suggests that exercise might enhance branching morphogenesis, which is an important precursor for nephrogenesis. In order to fully assess the relevance of the current results for fetal renal development, it's integral that the extent of branching is measured in fetuses. This could be done by immunostaining. For example, calbindin is a calcium binding protein expressed specifically within the UB of the developing kidney, so detection could highlight the spatial extent of UB branching at different time points in embryonic development (Cain et al. 2005). The edges of ureteric tips may also be labelled by highlighting the cap mesenchyme which can be detected by sine oculis-related homeobox 2 (SIX2) positive staining (Packard et al. 2013). Additionally, the experimental design of this chapter did not include a control exercised group, as the main question being addressed was how maternal exercise might serve as an intervention to prevent the adverse outcomes in offspring associated with maternal

obesity. However, given the current findings, it would also be interesting to assess UB branching and markers of renal morphogenesis in fetuses exposed to a control gestation with exercise. This would allow us to dissect the effects of maternal obesity and exercise on offspring renal development.

Although nephron endowment has been shown to be positively associated with the extent of UB branching (Cebrian et al. 2014; Awazu & Hida 2015), others have also shown that this is not always the case (Zhang et al. 2007). It will therefore also be important to count the number of nephrons, using the physical disector/fractionator combination (see chapter 4, section 4.3.2.3), in offspring exposed to a maternal control or obesogenic diet, with or without exercise after the completion of nephrogenesis. Assessing renal morphology throughout development and after nephrogenesis completion will allow us to determine firstly whether offspring exposed to maternal obesity have impaired renal morphogenesis which might explain the morphological changes in these offspring presented in chapter 4, and secondly allow us to determine whether maternal exercise alters renal morphogenesis in either offspring exposed to control or obese dams. Ultimately, the aim will be to determine whether exercise in obese dams may improve renal health in exposed offspring in later life.

Finally, a limitation of this study was that E19 fetuses were not sexed before the kidneys were pooled for analysis. Pooling males and females is likely to dilute any effects of the maternal environment, and so if anything this limitation emphasises the influence of maternal exercise on markers of offspring renal development. Nevertheless, in the future it will be important to separate male and female offspring to assess the potential effects of sex on renal development and morphology.

7.5.3 Conclusions

The results presented in this chapter demonstrate that exposure to maternal obesity with exercise during gestation increases a key gene integral for UB outgrowth in offspring, relative to offspring exposed to obesity alone, and that this may be associated with improved insulin levels in obese exercised dams. Maternal obesity with exercise also increased other markers of UB morphogenesis. Whilst no solid conclusions can be drawn from these findings regarding the nature of renal development in offspring, the results are intriguing and highlight key differences between offspring exposed to maternal obesity and offspring exposed to maternal obesity with exercise. Future studies will be integral to measure the extent of UB branching and nephrogenesis in offspring in this model. Ultimately, these studies will be conducted with a view towards assessing whether maternal exercise can improve the long-term renal health of offspring exposed to maternal obesity.

7.5.4 Summary

- E19 fetuses exposed to maternal obesity with exercise had increased renal *Gdnf* expression, a key gene required for UB branching. Maternal insulin levels correlated negatively with offspring *Gdnf* gene expression.
- Maternal exercise in obese dams increased markers of UB morphogenesis within the kidneys of exposed fetuses.
- Future studies will be required to determine whether maternal obesity impairs renal morphogenesis and whether maternal exercise in obese dams improves any such alterations.

Chapter 8. General Discussion

The aim of this thesis was to investigate the impact of maternal obesity on offspring kidney morphology and later life renal health. To date, only one study in humans has investigated the effect of maternal obesity on the offspring kidney, demonstrating an association between maternal obesity and renal dysplasia/aplasia and obstructive uropathy in children (Hsu et al. 2014). No epidemiological studies to date, have investigated the impact of exposure to maternal obesity on longer term renal health. CKD prevalence has increased rapidly in recent years concurrently with rising metabolic syndrome rates. In the UK and US, the majority of women of child-bearing age are now overweight or obese (Richter et al. 2015). It's probable that maternal obesity, by directly programming the offspring kidney and by increasing the risk of the metabolic syndrome in exposed individuals (which is a significant driver of CKD) is an important contributor to the current CKD disease burden. However this has yet to be investigated. In addition to human studies this necessitates the use of animal models both to characterise the effects of maternal obesity on the long-term health of the offspring kidney, and to explore potential mechanisms involved in the programming of the offspring kidney by maternal obesity. Ultimately these investigations may aid in the establishment of an intervention for maternal obesity which might decrease the burden of CKD in future generations.

8.1 Maternal diet-induced obesity and the offspring renal phenotype

This thesis assessed the effect of maternal diet-induced obesity throughout gestation and lactation on the offspring renal morphology and indicators of renal health from fetal life to adulthood. Figure 8.1 shows a summary of the main findings.

Male offspring

Fetal life
- Inta-uterine growth restriction
- *Renal programming*
  - *Increase in genes and proteins important for renal morphogenesis*

(In offspring exposed to maternal obesity with exercise)

Neonatal to weaning
- Accelerated catch-up growth
- Metabolic programming
  - ↑ *Insulin, glucose, cholesterol, triglycerides*
- Renal programming
  - *Renal catch up growth*
  - *Glomerular hypertrophy*

Maternal obesity (45% fat and sucrose)

Young adulthood
- Metabolic programming
  - ↑*insulin, leptin*
- Cardiovascular programming
  - ↑*blood pressure and heart rate*

Mature adulthood
- Renal programming
  - *Increased lipid deposition*
  - *Gene expression indicative of inflammation and fibrosis*
  - *Glomerulosclerosis*
  - *Tubulointerstitial fibrosis*

(Exacerbated by post-weaning obesity)

Figure 8.1. **A summary of the main findings of this thesis**

The most significant morphological finding was that offspring of obese dams showed glomerular hypertrophy from weaning (chapter 4) and until adulthood (chapter 5). Glomerular hypertrophy is often associated with nephron deficit and the Brenner hypothesis postulates that the two characteristics can be inter-linked; a nephron deficit forces each individual nephron to undergo hyperfiltration in order to maintain overall renal GFR levels, leading to hypertrophy (Mackenzie & Brenner 1995; Brenner & Mackenzie 1997). Consistently, animal models of maternal under-nutrition and diabetes have shown that exposed offspring have fewer nephrons, develop glomerular hypertrophy and go on to develop renal dysfunction and disease in later life (see Table 1.2 in chapter 1). However, nephron deficit is not a required pre-requisite for early glomerular hypertrophy. Studies of maternal diabetes induced by STZ have shown that exposed offspring have the same number of nephrons as control animals (Rocha et al. 2005; Magaton et al. 2007), but develop glomerular hypertrophy nonetheless along with renal dysfunction, suggesting glomerular hypertrophy is an independent predictor of later life renal disease. The finding that offspring exposed to maternal obesity in the current study had glomerular hypertrophy therefore suggested that these offspring might be predisposed for renal disease in later life.

Offspring exposed to maternal obesity showed no evidence of renal damage (as assessed by fibrosis staining) in young adulthood (chapter 5). However, consistent with the observed early life changes in morphology, by later adulthood offspring of obese dams showed extensive evidence of renal damage (chapter 6). Six month old offspring exposed to maternal obesity had a modest increase in intra-renal lipid deposition, and showed modest up-regulation of genes indicative of inflammation and fibrosis. Importantly, there was a strong effect of maternal obesity on offspring glomerulosclerosis and tubulointerstitial fibrosis. This is a striking finding for a number of reasons. Firstly, glomerulosclerosis and tubulointerstitial fibrosis are final convergent characteristics of a variety of renal pathologies leading to ESRF (Ma & Fogo 2003; Zeisberg & Neilson 2010), demonstrating that exposure to maternal obesity led to extensive renal damage in offspring. Secondly, C57BL6 mice are known to be fairly resistant to renal injury (Breyer et al. 2009), so the fact that significant fibrosis was seen in offspring of obese dams in this strain demonstrates that maternal obesity is a powerful promoter of adverse renal programming. Thirdly, extensive fibrosis was seen in offspring exposed to maternal obesity by 6 months of age, which is still a fairly young age given that C57BL/6 mice can live for around 2 years. This emphasises the impact of maternal obesity on offspring renal health.

Despite the glomerular hypertrophy and fibrosis observed in offspring exposed to maternal obesity, there was no evidence that renal function was affected in these animals in adulthood. In young adulthood, offspring showed normal indicators of renal function including urine albumin levels (indicating that the glomerular filtration barrier was intact), serum creatinine levels (suggesting overall

renal GFR rates were normal) and ions (suggesting renal ion handling was intact) (chapter 5). In later adulthood, offspring exposed to maternal obesity also showed no changes in blood urea nitrogen (BUN) or cystatin C (chapter 6), suggesting that overall renal GFR was intact. The fact that a decrease in renal function was not seen alongside renal damage in offspring exposed to maternal obesity at 6 months of age is not entirely surprising. The kidney is a resilient organ, able to adapt to challenges that promote damage in order to cope with functional demands. Glomerular and tubule damage leading to fibrosis, eventually results with the loss of functional nephrons. However, remaining nephrons are able to undergo adaptations including intra-glomerular hypertension, hyperfiltration and hypertrophy (Kriz & LeHir 2005; Schnaper 2014). These changes maintain overall GFR but also increase the vulnerability of remaining nephrons to damage, initiating a vicious cycle leading to further nephron loss. Once a certain threshold of nephron loss is reached the kidney can no longer cope with demand and GFR declines rapidly progressing to ESRF. It's possible that at 6 months of age, the damage within the kidneys of offspring exposed to maternal obesity had not yet reached the threshold beyond which total renal function begins to deteriorate. In the future it will be interesting to measure GFR in offspring beyond 6 months of age, to fully characterise the progression of renal damage in offspring exposed to maternal obesity.

8.2 The effect of an offspring obesogenic diet in combination with maternal obesity on offspring renal health.

Chapter 6 investigated the impact of a post-weaning obesogenic diet in combination with maternal obesity on offspring renal health. It was found that post-weaning obesity independently increased markers of renal damage, including intra-renal lipid deposition, genes relating to renal inflammation and fibrosis, and glomerulosclerosis and tubulointerstitial fibrosis. This is consistent with epidemiological findings demonstrating that obesity and associated pathologies including hypertension and hyperglycaemia are important causes of CKD (Bagby 2004; Maric-Bilkan 2013). Importantly, a post-weaning obesogenic diet accentuated the adverse effects of a maternal obesogenic diet on the offspring kidney, with offspring exposed to both conditions showing the most extensive fibrosis. These findings have important implications regarding the aetiology of the current CKD epidemic; they corroborate epidemiological findings, demonstrating that being obese is a major risk factor for CKD, but they also uncover a key role of maternal obesity in renal programming. The current findings suggest that CKD prevalence might be increasing rapidly at present in a similar manner to the exponential increase in obesity and type 2 diabetes rates seen in recent years, i.e. poor health in one generation leads to a propagation and amplification of poor health in the next, promoting a vicious cycle. Given the fact that both obesity rates in the general population, and obesity rates in women of child-bearing age are set to continue to increase, this suggests that the burden of CKD will grow in the coming years. This highlights a need for an effective intervention for maternal obesity, to halt the vicious cycle and decrease the burden of renal diseases in future generations.

8.3 Possible mechanisms programming adverse renal health in offspring exposed to maternal obesity.

There are many factors associated with maternal obesity that could program the offspring kidney leading to poor renal health in later life. Our laboratory has shown that obese dams are glucose intolerant, hyperinsulinaemic and have indicators of poor placental function during pregnancy, and that these factors are improved by maternal exercise in the obese dams independent of reduction in maternal weight (Fernandez-Twinn et al. 2017). In chapter 7, it was demonstrated that markers of renal morphogenesis were enhanced in fetuses exposed to maternal obesity with exercise, suggesting that exercise might ameliorate renal programming by maternal obesity (although further research needs to be done to confirm this). This therefore implies that insulin, glucose and placental insufficiency could be important mediators of renal programming in offspring exposed to maternal obesity. There are also other, as yet unexplored, factors which could lead to renal programming by maternal obesity including glucocorticoid overexposure. These factors could program the offspring kidney directly by influencing renal development.

8.3.1 Potential maternal programming factors

8.3.1.1 Hyperinsulinaemia and glucose intolerance

Maternal hyperinsulinaemia is known to result from maternal hyperglycaemia, which promotes excess glucose transport across the placenta, leading to fetal hyperglycaemia and hyperinsulinaemia. Consistent with this notion, our laboratory has recently showed that fetuses exposed to maternal obesity have higher levels of pancreatic insulin (unpublished). Glucose has been demonstrated to have a strong effect on offspring kidney development. Glucose has been shown to have both inhibitory (Hokke et al. 2013) and stimulatory (Zhang et al. 2007) effects on ureteric bud branching, to decrease nephrogenesis (Nehiri et al. 2008; Tran et al. 2008; Hokke et al. 2013), and to increase apoptosis within the developing kidney (Tran et al. 2008; Lin et al. 2013). Glomerular hypertrophy has also been observed in offspring exposed to early life hyperglycaemia (Rocha et al. 2005; Magaton et al. 2007; Rocco et al. 2008), consistent with findings in offspring exposed to maternal obesity in the current thesis. Maternal hyperinsulinemia is also known to promote excess lipid availability to the fetus (Catalano & Hauguel-De Mouzon 2011). Potential direct effects of increased lipid availability on the kidney have not (yet) been demonstrated. One study did show that a maternal high fat diet induced maternal hyperinsulinaemia independent of changes in maternal glucose tolerance or weight during late pregnancy and this led to an increase in nephron endowment in exposed offspring (Hokke et al. 2016). Chapter 7 showed that decreasing maternal insulin levels in obese dams by maternal exercise

enhanced markers of renal morphogenesis and is therefore consistent with the notion that maternal hyperinsulinaemia/hyperglycaemia has a negative impact on renal development.

8.3.1.2 Placental insufficiency

Placental insufficiency reduces the delivery of oxygen and nutrients to the developing fetus resulting in IUGR (Krishna & Bhalerao 2011). Studies of placental insufficiency caused by uterine artery ligation have demonstrated that offspring develop fewer nephrons (Sanders et al. 2004; Wlodek et al. 2008) and glomerular hypertrophy (Sanders et al. 2004). These outcomes appear to be very similar to those resulting from models of maternal under-nutrition and protein restriction (see Chapter 1, section 1.5.1), most likely because all these models reduce nutrient delivery to the fetus. Additionally, studies of maternal hypoxia have shown an increase in apoptosis and autophagy within exposed fetal kidneys (Xia et al. 2015) and a reduction in neonatal nephron number (Gonzalez-Rodriguez et al. 2013). Together these findings suggest that placental insufficiency, by decreasing nutrient and oxygen delivery to the fetus, impairs renal morphogenesis. In our model, a role for placental insufficiency in offspring renal programming by maternal obesity is supported by the fact that offspring were growth restricted at birth, the kidney underwent catch up growth and glomerular hypertrophy was observed. Furthermore, restoring placental health by maternal exercise in obese dams increased markers of renal morphogenesis in fetal offspring.

8.3.1.3 Glucocorticoids

A variety of maternal environmental perturbations leading to fetal growth restriction are associated with high fetal glucocorticoid levels (Seckl & Holmes 2007), suggesting that glucocorticoid over-exposure might be a common programming mechanism in IUGR. One way the fetus can be over-exposed to glucocorticoid is by the downregulation of 11β-HSD2 in the placenta, an enzyme which converts active glucocorticoid to its inactive form. Placental 11β-HSD2 is downregulated in both epidemiological (Nuyt 2008) and animal studies (Langley-Evans et al. 1996) complicated by IUGR. Glucocorticoid over-exposure, like other models leading to IUGR, decreases nephron number in offspring (Wintour et al. 2003; Dickinson et al. 2007) and can lead to glomerular hypertrophy (Wintour et al. 2003). It has also been demonstrated that offspring exposed to synthetic glucocorticoid during mid-gestation had an up-regulation of genes involved in the inhibition of UB branching in fetal life, and fewer nephrons in later life (Dickinson et al. 2007), demonstrating a direct effect of glucocorticoid on renal development. It's possible that glucocorticoid overexposure could be involved in the programming of the offspring kidney in our model and this is a subject that warrants further investigation.

It's important to note that the potential programming factors discussed above are not mutually exclusive. For example placental 11β-HSD expression has been shown to be decreased in placental insufficiency (Zhang et al. 2016). Ultimately, the potential programming factors described above likely lead to alterations in the local renal developmental environment. This could promote epigenetic changes in genes controlling kidney morphogenesis, leading to permanent modifications and predisposing the organ for damage in later life. In support of this, there have been a few studies demonstrating an association between an adverse early life environment and/or altered renal development with renal epigenetic changes. Baboon fetuses exposed to maternal nutrient restriction showed global methylation changes in their kidneys (Unterberger et al. 2008). Rats exposed to uteroplacental insufficiency showed a reduction in nephron number at term as well as increased mRNA levels of apoptotic factors including p53. Increased p53 expression could be explained by decreased CpG methylation at its promoter site (Pham et al. 2003). Of particular interest to the current thesis, the ability of Angiotensin II to induce ureteric bud branching (Song et al. 2010), and *Gdnf* expression have been shown to be controlled by histone deacetylase activity (Sylviva & Samir 2016). This raises the possibility that fetuses exposed to maternal obesity in the current thesis showed a trend towards reduced *Gdnf* levels due to epigenetic modulation. Further research is needed to distinguish which factors associated with maternal obesity are important for offspring renal programming, and to establish the mechanisms linking these factors to offspring renal damage.

8.3.2 Potential indirect programming effects

It's important to also consider that factors programmed in offspring by maternal obesity could indirectly undermine kidney health in later life. In this thesis, young adult offspring exposed to maternal obesity showed increased blood pressure and insulin levels, which are known to be important mediators of renal damage. The potential mechanisms leading to renal damage by these factors are discussed below.

8.3.2.1 Hypertension

Systemic hypertension can promote dilatation of the afferent arteriole, leading to an increase in the pressure within glomerular capillaries (Click et al. 1979; D'Agati et al. 2016) and thereby leading to glomerular hyperfiltration and hypertrophy (Kriz & LeHir 2005; D'Agati et al. 2016). These changes put the podocytes under excessive strain, leading to a vicious cycle of podocyte loss, compensatory podocyte hypertrophy and detachment of podocytes from the GBM (Kriz & LeHir 2005). The loss of GBM integrity then leads to glomerulosclerosis and proteinuria, and subsequently tubulointerstitial

fibrosis (see chapter 1, section 1.2.1.2 for a detailed description of how hypertension promotes renal damage). The observations that offspring exposed to maternal obesity in this thesis had increased glomerular hypertrophy in young life and renal fibrosis by mature adulthood are consistent with the notion that increased blood pressure played a key role in initiating renal damage.

8.3.2.2 Hyperinsulinaemia/insulin resistance

Insulin is a powerful mediator of renal damage. As discussed in chapter 1, section 1.2.1.1.2, it has been demonstrated that hyperinsulinaemia is an independent predictor of CKD in humans (Bagby 2004; De Vries et al. 2014). In short, hyperinsulinaemia leads to hyperfiltration and glomerular hypertrophy via endothelial dependent vasodilatation, thereby increasing shear stress within glomeruli (Bagby 2004; Hale & Coward 2013). Additionally, insulin can induce direct damage to glomeruli by promoting mesangial cell ECM deposition (Abrass et al. 1994), loss of mesangial cell contractile function (D'Agati et al. 2016), and podocyte specific insulin resistance (Hale & Coward 2013; De Vries et al. 2014) (see chapter 1, section 1.2.1.1.2 for a more detailed description). These changes lead to glomerulosclerosis and proteinuria. The observations that offspring exposed to maternal obesity in the current study showed glomerulosclerosis, glomerular hypertrophy and had increased intra-renal lipid deposition are consistent with a role for hyperinsulinaemia/insulin resistance in inducing renal damage in these offspring.

Figure 8.2 shows the proposed potential mechanisms leading to renal damage in offspring exposed to maternal obesity in the current model.

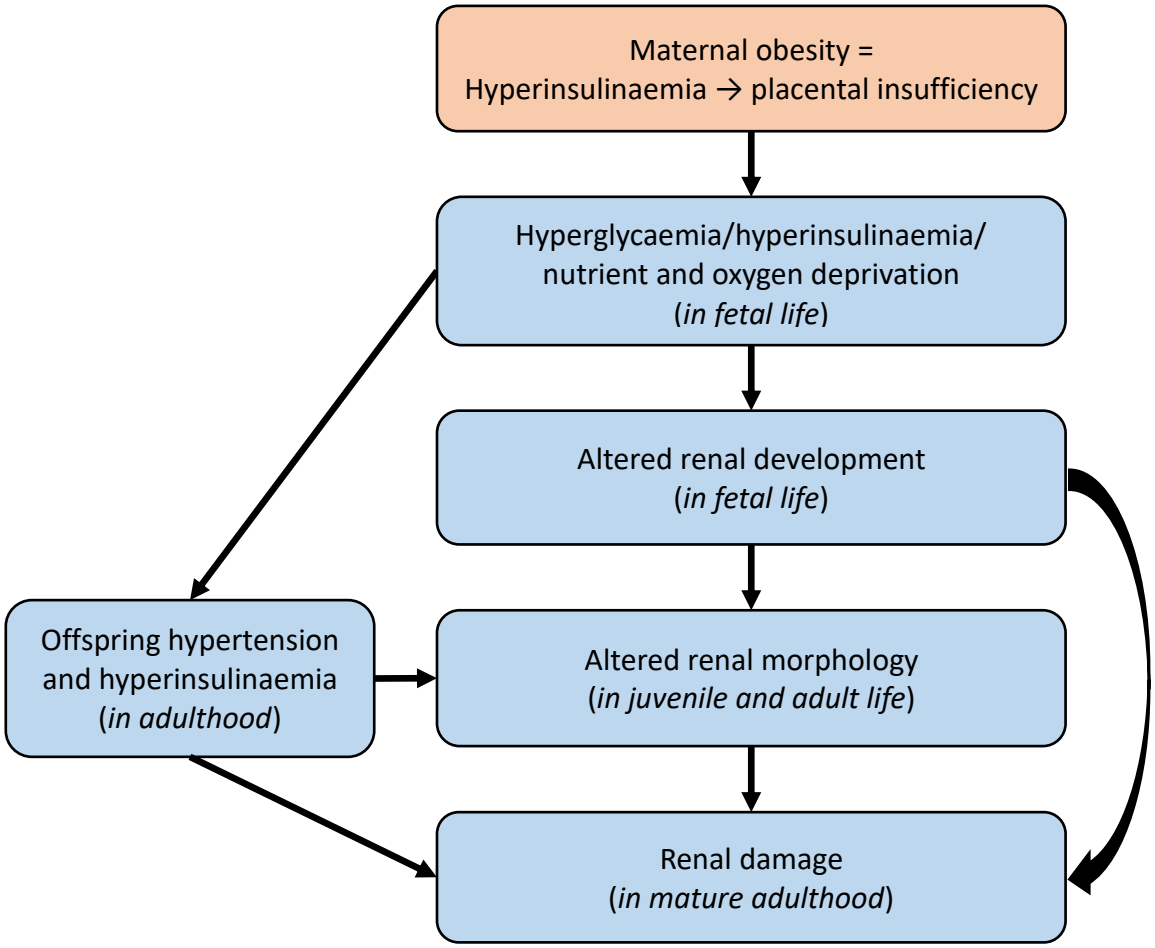

Figure 8.2. **Diagram of the simplified proposed mechanism leading to offspring renal damage following exposure to maternal obesity.**

8.4 Limitations and future directions

The findings in this thesis suggest that maternal obesity might play an important role in adverse renal programming leading to later life renal damage. However, these findings also raise many questions which will be important to address in the future if an effective intervention for maternal obesity that ameliorates the burden of renal diseases in coming generations is to be implemented. These, along with the limitations of this work, are discussed below.

8.4.1 What is the effect of maternal obesity on the placenta and fetal nutrient delivery?

Our laboratory has already showed that maternal obesity increases lipid deposition and HIF1A expression in the placenta, and that maternal exercise, likely through reducing maternal insulin levels, rescues these changes (Fernandez-Twinn et al. 2017). These findings, along with IUGR in offspring of obese dams, strongly suggest that obese dams have placental insufficiency, which would decrease the delivery of oxygen and nutrients to the developing fetus. Maternal obesity with GDM is also known to induce fetal hyperglycaemia leading to fetal hyperinsulinaemia, which is also observed in offspring exposed to maternal obesity in our model. Characteristics in offspring exposed to maternal obesity such as increased blood pressure and hyperinsulinaemia, and renal effects such as fibrosis and glomerular hypertrophy are consistent with programming by either placental insufficiency or fetal hyperglycaemia/hyperinsulinaemia. Therefore, in order to better characterise the mechanisms leading to renal programming in offspring, it will be integral to accurately measure placental function. As described in chapter 7, section 7.5.2, this could be done by measuring fetal and placental blood flow by doppler ultrasound, and by tracking specific nutrients from the maternal to the fetal circulation.

8.4.2 Are there any programmed changes to renal development due to maternal obesity?

The findings in this thesis demonstrated that by weaning, offspring renal morphology was altered by exposure to maternal obesity. Additionally, in fetal life offspring exposed to maternal obesity with exercise showed increases in gene and protein expression of factors important for UB branching when compared to offspring exposed to obesity alone. These findings suggest that maternal obesity might adversely program offspring renal development. A limitation of this thesis was that renal development, by studying renal morphology, was not assessed in fetal life. In the future it will be important to measure UB branching in fetuses exposed to maternal obesity and to assess other potential changes in the developing kidney such as apoptotic activity. Both UB branching, and

apoptotic activity have been shown to be altered within the kidneys of growth restricted offspring exposed to maternal diabetes induced by STZ (Tran et al. 2008; Hokke et al. 2013; Lin et al. 2013). In addition to determining the structure of the developmental kidney, it will be important to better characterise gene and protein expression changes that accompany any changes in morphology. Ultimately, the epigenome of the developing kidney could be characterised to assess whether epigenetic alterations may underpin any changes in developmental morphology in offspring exposed to maternal obesity. Epigenetic alterations in the fetal/neonatal kidneys may also help to explain the predisposition to renal ectopic lipid deposition and fibrosis in adult offspring exposed to maternal obesity. Notably, our laboratory has recently shown that genes relating to fatty acid metabolism are altered within the hearts of mouse fetuses exposed to maternal obesity (Loche 2016). It is therefore possible that fatty acid metabolism pathways are also programmed in the kidney and it's possible that this is one factor driving the increased intra-renal lipid deposition in offspring exposed to maternal obesity. Additionally, it has been demonstrated that histone modifications control the transcription of genes leading to fibrosis such as *Fn1* in diabetic animals (Sun et al. 2017). However, whether such genes are epigenetically altered by exposure to early life adverse environments, such as maternal obesity, remains unexplored. These investigations will help to uncover the mechanisms linking maternal obesity to offspring renal damage.

8.4.3 What is causing glomerular hypertrophy in offspring of obese dams?

Glomerular hypertrophy was observed in 3 week old mice exposed to maternal obesity and was present until adulthood (8 weeks of age). Glomerular hypertrophy has been shown to be a central feature of hypertensive nephropathy preceding glomerulosclerosis (Hughson et al. 2014). In models of nephron mass reduction, glomerular hypertrophy also precedes renal damage (Kriz & LeHir 2005). In this setting, glomerular hypertrophy is due to compensatory adaptation of remaining nephrons to maintain overall GFR. However, compensatory glomerular hyperfiltration and hypertrophy leaves these nephrons susceptible to damage and this is why glomerular hypertrophy is commonly seen before CKD (Hodgkins & Schnaper 2012; Schnaper 2014). Additionally, in obese patients glomerular hypertrophy is thought to precede focal segmental glomerulosclerosis (Helal et al. 2012). Consistently, type 2 diabetes and hypertension (central features of the metabolic syndrome) promote renal disease through the same mechanisms, i.e. increased GFR and glomerular hypertrophy leading to proteinuria and focal segmental glomerulosclerosis as well as tubular damage (Maric-Bilkan 2013). Mouse models of diabetic nephropathy have also demonstrated that haemodynamic changes leading to glomerular hypertrophy precede mesangial matrix expansion (Breyer et al. 2009). Therefore, in a variety of clinical settings in both humans and animals, glomerular hypertrophy is a unifying factor predicting renal

damage. It is therefore important to address the question of what is causing glomerular hypertrophy in offspring exposed to maternal obesity in our model. Determining the causes might direct us towards an effective intervention. Our laboratory has demonstrated that at 3 weeks of age, mice exposed to maternal obesity show gene expression in the heart indicative of pathologic hypertrophy (Blackmore et al. 2015). Therefore, measuring markers of renal pathological hypertrophy such as calcineurin (a calcium-dependent phosphatase) in neonatal mice could highlight whether glomerular hypertrophy is directly programmed by maternal obesity or is secondary to physiological factors such as hypertension (C. R. Williams et al. 2014). Hypertension was observed in 8 week old offspring exposed to maternal obesity, but it is not technically feasible to measure blood pressure in 3 week old mice by tail cuff plethysmography. Miniature telemetry systems which will allow blood pressure recordings in very immature rodents are currently being developed (Zayachkivsky et al. 2015). Therefore, in the future, it may be possible to utilise telemetry to assess blood pressure in 3 week old mice. This might highlight a potential contribution of blood pressure to glomerular hypertrophy.

8.4.4 What is the aetiology of renal damage in offspring of obese dams?

Offspring of obese dams showed increased blood pressure, insulin levels and a tendency towards increased fat deposition which could, along with other unknown factors, act as primary factors driving the renal damage observed in these animals. It's important to address the question of how renal damage progresses in offspring exposed to maternal obesity. In most cases of CKD, it's thought that glomerular damage precedes tubular damage (Kriz & LeHir 2005). However there can be subtle differences in disease progression depending on the initiating factors. The increase in blood pressure and insulin levels in offspring exposed to maternal obesity would be consistent with the so-called "degenerative" pathway of renal damage. In this pathway, glomerular hypertension and metabolic disturbances challenge podocytes and promote their effacement from the GBM. This decreases the density of functional podocytes, putting excessive strain on them as described in section 8.3.2.1. Eventually the GBM barrier is breached and proteins can pass freely into the bowman's space. Parietal cells of the Bowman's capsule can also then attach to naked areas of the GBM and eventually areas of the glomerular tuft attach to areas of the Bowman's capsule. Since this adhesion of the glomerular tuft leads proteins to be misdirected into the interstitium instead of the bowman's space, fibroblasts attempt to limit fluid entry into the interstitium and this results in crescent formations in the bowman's space as well as hyaline crystals in the tuft (Kriz & LeHir 2005). This mechanism of renal damage would be consistent with the increased blood pressure and insulin, and glomerular hypertrophy, in offspring of obese dams in our model. Nevertheless, it would be interesting to look at defining features of this pathway in animals before 6 months of age (by which time glomerulosclerosis

is evident). For example, electron microscopy could be utilised to visualise podocyte foot process effacement (Deegens et al. 2008). The presence of crescents and hyaline crystals could also be assessed by staining and imaging. In the degenerative pathway, glomerular damage causes progressive albuminuria which is a major promoter of tubulointerstitial fibrosis, an outcome which was also displayed by 6 month old mice exposed to maternal obesity in our model. One limitation of this thesis was that albuminuria was not assessed in offspring at 6 months of age. Assessing albuminuria in offspring before and until 6 months of age will be important in determining the aetiology of renal damage.

8.4.5 How does maternal obesity effect the offspring kidney in older age?

A limitation of this thesis was that kidneys were only assessed in offspring exposed to maternal obesity until 6 months of age. This is equivalent to around 30 years in humans (Ferando et al. 2016). It would therefore be valuable to assess kidneys in animals into middle and old age, especially since the findings at 6 months suggest that age may be an important catalyst for renal damage programmed by maternal obesity. Notably, offspring of obese dams showed increased fibrosis relative to control animals at 6 months of age. As described in section 8.1, nephron damage leading to fibrosis initiates a vicious cycle of further nephron loss. Functional nephrons can compensate for lost nephrons and maintain the functional requirements of the kidney up until a certain point, but beyond this threshold overall GFR beings to diminish leading to CKD and eventually ESRF. Studies of maternal diabetes induced by STZ during gestation emphasise that ageing may be important in unmasking the effects of maternal programming in the offspring kidney (Rocha et al. 2005; Nehiri et al. 2008). Notably, rats exposed to maternal diabetes showed an accelerated decline in GFR after 6 months of age until the end of the study (12 months of age). The most extensive proteinuria was also seen in these animals at 12 months of age (Nehiri et al. 2008). Therefore, it may be insightful to measure GFR and albuminuria in offspring in our model beyond 6 months of age to fully characterise the progression of renal damage and to assess the presence of CKD due to exposure to maternal obesity.

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8.4.6 Is maternal hyperinsulinaemia an ideal target for intervention in maternal obesity to prevent renal programming?

Our laboratory has previously shown that maternal exercise during pregnancy improves insulin levels in obese dams and decreases insulin levels and improves insulin sensitivity in exposed offspring (Fernandez-Twinn et al. 2017). More recently, we have also shown that offspring exposed to maternal

obesity with exercise demonstrate improved cardiac function at 8 weeks of age relative to offspring exposed to obesity alone (unpublished). These findings suggest that reducing maternal insulin levels during pregnancy might also be an effective interventional strategy to prevent adverse renal outcomes in offspring exposed to maternal obesity. Indeed in chapter 7 of this thesis, it was demonstrated that an important gene involved in UB branching was negatively correlated with maternal insulin levels and increased in offspring exposed to maternal obesity with exercise. In the future it will be important to address the aforementioned research questions in offspring exposed to maternal obesity with exercise to establish whether maternal hyperinsulinaemia is a central promoter of renal damage in offspring and therefore an ideal target for intervention.

8.5 Concluding remarks

The aim of this thesis was to characterise the effects of exposure to maternal obesity throughout gestation and lactation on the offspring kidney. The most important findings were that males exposed to maternal obesity showed glomerular hypertrophy from a very young age, and developed renal fibrosis in adulthood. A post-weaning obesogenic diet also exacerbated the renal damage observed in offspring exposed to maternal obesity. Future work will focus on determining the maternal factors associated with obesity that are important for renal programming in offspring, with view towards designing an effective intervention that may reduce the burden of renal diseases in future generations.

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