Glucose intolerance in pregnancy: Consequences for offspring kidney development and adult renal health

A thesis submitted for the degree of
Doctor of Philosophy

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ABSTRACT

The prevalence of glucose intolerance in pregnancy is increasing worldwide. Both pre-existing Type 2 diabetes and gestational diabetes have known health implications for offspring yet few studies have assessed offspring renal development and function. In rodents, offspring exposed to persistent hyperglycaemia have a nephron deficit. However, this model does not reflect the typical clinical condition and is associated with fetal growth restriction that may confound the effect on nephron endowment. In humans, estimation of total nephron number is only feasible in autopsy tissue. Studies assessing fetal kidney volume, as a proxy of renal sufficiency, in women with glucose intolerance in pregnancy are sparse and report equivocal findings.

The studies presented in this thesis investigated offspring kidney development in mouse models of both pre-gestational Type 2 diabetes and gestational diabetes. It was hypothesised that offspring of pregnancies complicated by glucose intolerance would be macrosomic, yet exhibit deficits in nephron endowment. It was further hypothesised that offspring would be overweight and glucose intolerant in adulthood with perturbed renal function and morphology. Additionally, this thesis aimed to further our understanding of how gestational diabetes affects human fetal kidney size. It was hypothesised that fetuses of women with gestational diabetes would have smaller kidney volumes.

Three major studies were undertaken. In the first experimental study, C57BL6 mice were fed a high fat diet (HFD, 21% fat) prior to and throughout pregnancy to model obesity-associated pre-gestational Type 2 diabetes. Compared with mice fed a normal fat diet (NFD, 6% fat), mice fed a HFD were overweight and glucose intolerant prior to pregnancy, but unfortunately not in late gestation. Offspring of HFD dams were macrosomic at embryonic day (E) 18.5 and had a tendency to be heavier at postnatal day (PN) 21. Using unbiased stereology, the kidneys of HFD offspring were found to contain 20-25% more nephrons than kidneys of NFD offspring. Nephron number was increased in HFD offspring regardless of maternal weight and glucose profiles prior to pregnancy. Offspring of HFD dams displayed mild fasting hyperglycaemia at 9 months of age, but surprisingly had normal body weight, glucose tolerance and body composition. Renal function and morphology, measured by transcutaneous clearance of FITC-sinistrin and stereology respectively, were also normal at 9 months of age.

In the second experimental study, C57BLKS/J leptin receptor deficient mice (*Lepr*$^{db/+}$) were characterised to assess their suitability as a model of gestational diabetes. Leptin is a hormone produced primarily by adipocytes that acts on receptors in the hypothalamus to regulate energy balance by decreasing food intake and increasing energy expenditure. Compared with wildtype

(+/+) mice, leptin resistant *Lepr*$^{db/+}$ mice were overweight and glucose intolerant prior to pregnancy, with glucose profiles exacerbated in late gestation. *Lepr*$^{db/+}$ mice were therefore a model of pre-gestational Type 2 diabetes rather than gestational diabetes as anticipated. Offspring of *Lepr*$^{db/+}$ dams were of normal body weight at E18.5 and PN21 and exhibited a 15% nephron deficit which was evident before birth.

The opposing effects on nephron endowment in offspring of HFD and *Lepr*$^{db/+}$ dams are likely reflective of differences in maternal phenotypes during the period of kidney development. The increased nephron endowment in HFD offspring was not attributable to maternal obesity or glucose intolerance and is possibly be due to constituents of the HFD promoting nephrogenesis. Conversely, the deficit in nephron endowment in *Lepr*$^{db/+}$ offspring was best predicted by maternal glucose intolerance in late gestation. Additional studies are required to identify the molecular mechanisms that lead to increased and decreased nephron endowment in these two models.

The third study compared fetal biometry and fetal kidney size in a prospective cohort study of women with and without gestational diabetes. Fetal biometry and kidney volume (calculated using the approximation of a prolate ellipsoid) were measured by obstetric ultrasound at 32-34 weeks gestation in women identified with (n=36) and without (n=42) gestational diabetes by routine testing at 24-28 weeks. In this preliminary study, estimated fetal weight and birth weight were lower in women with gestational diabetes. Amniotic fluid index and fetal cerebral vascular resistance were also lower, although values were within the normal clinical range. Despite these outcomes, fetal kidney volume in offspring of women with gestational diabetes was similar to those of normal control pregnancies.

As maternal diabetes is typically associated with neonatal macrosomia, it is unclear why lower fetal weight was observed in the human gestational diabetic cohort. However, a possible explanation is low-level placental dysfunction. The preliminary findings from the prospective cohort study suggest that hyperglycaemia does not affect fetal kidney volume. Analysis of fetal kidney volume in a larger population incorporating maternal ethnicity and degree of glucose intolerance, as well as in women with longer periods of hyperglycaemia (such as in women with early gestational diabetes and undiagnosed pre-gestational Type 2 diabetes) is required.

DECLARATION OF AUTHORSHIP

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Stacey Hokke

Date

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Conference presentations

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HOKKE, S. N., ARMITAGE, J. A., PUELLES, V.G., BERTRAM, J. F. & CULLEN-MCEWEN L. A. A Western diet leading to maternal glucose intolerance and obesity elevates offspring nephron

endowment without adverse effects on adult renal function. Poster presentation at the American Society of Nephrology (ASN) Annual Meeting, Philadelphia, U.S.A., 2014.

HOKKE, S. N., ARMITAGE, J. A., PUELLES, V.G., FONG, K., BERTRAM, J. F. & CULLEN-MCEWEN L. A. Impaired glucose tolerance in pregnancy reduces nephron endowment in offspring. Oral presentation at the Australasian Diabetes in Pregnancy Society (ADIPS) Annual Scientific Meeting, Queenstown, New Zealand, 2014.

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ABBREVIATIONS, EQUATIONS AND SYMBOLS

AC	Abdominal circumference
ACOG	American College of Obstetricians and Gynecologists
ADA	American Diabetes Association
ADIPS	Australasian Diabetes in Pregnancy Society
AFI	Amniotic fluid index
AGA	Appropriate for gestational age
AIHW	Australian Institute of Health and Welfare
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
AUC	Area under the curve
BABB	Benzyl alcohol/benzyl benzoate
BB	Bio-breeding rat
BMI	Body mass index
BMP4	Bone morphogenetic protein 4
BOS	Birthing Outcomes System
BPD	Bi-parietal diameter
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CCD	Charge-coupled device
CKD	Chronic kidney disease
DAB	Diaminobenzidine
DEXA	Dual energy X-ray absorptiometry
DNA	Deoxyribonucleic acid
Dock7	Dedicator of cytokinesis protein 7
DPX	Dibutyl polystyrene xylene
E	Embryonic day
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EFW	Estimated fetal weight
EGF	Epidermal growth factor
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbant assay
eRPF	Effective renal plasma flow
ESRD	End stage renal disease
FAT	Fatty acid translocase
FABP	Fatty acid binding protein
FATP	Fatty acid transport protein
FFA	Free fatty acids
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FL	Femur length
GCT	Glucose challenge test
GDNF	Glial cell-line derived neurotrophic factor

GFR	Glomerular filtration rate
GFRα1	Glial cell-line derived neurotrophic factor family receptor α1
GLUT	Glucose transporter
GTT	Glucose tolerance test
H_2O_2	Hydrogen peroxide
HAPO	Hyperglycemia and Adverse Pregnancy Outcome
HbA1c	Glycosylated haemoglobin
HC	Head circumference
HFD	High fat diet
HFD HBW	High fat diet, high body weight
HFD IGT	High fat diet, impaired glucose tolerance
HFD NBW	High fat diet, normal body weight
HFD NGT	High fat diet, normal glucose tolerance
HGF	Hepatocyte growth factor
HRP	Horseradish peroxidase
IADPSG	International Association of Diabetes and Pregnancy Study Groups
IGF	Insulin-like growth factor
IGF-IR	Insulin-like growth factor 1 receptor
IGF-IIR	Insulin-like growth factor 2 receptor
IgG	Immunoglobulin G
IGT	Impaired glucose tolerance
IL-6	Interleukin 6
IQR	interquartile range
IR	Insulin receptor
LED	Light-emitting diode
Lepr	Leptin receptor
LGA	Large for gestational age
LLQ	Left lower quadrant
LUQ	Left upper quadrant
LUSCS	Lower uterine segment Caesarean section
MCA PI	Middle cerebral artery pulsatility index
$MgCk_2$	Magnesium chloride
$MnCl_2$	Manganese chloride
NaOH	Sodium hydroxide
NaCl	Sodium chloride
NFD	Normal fat diet
NFD NBW	Normal fat diet, normal body weight
NFD NGT	Normal fat diet, normal glucose tolerance
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGT	Normal glucose tolerance
NIH	National Institute of Health
NOD	Non-obese diabetic mouse
OGTT	Oral glucose tolerance test
OPT	Optical Projection Tomography
PBS	Phosphate-buffered saline

PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PN	Postnatal day
PNA	Peanut Agglutinin
RANZCOG	Royal Australasian College of Obstetrics and Gynaecology
RCPA	Royal College of Pathologists Australia
RLQ	Right lower quadrant
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RUQ	Right upper quadrant
SD	Standard deviation
SEM	Standard error of the mean
SGA	Small for gestational age
SNAT	Sodium-coupled neutral amino acid transporter
SSF	Section sampling fraction
STZ	Streptozotocin
TBE buffer	Tris-Borate-EDTA buffer
TBS	Tris-buffered saline
TBS-Tx	Tris-buffered saline-Triton X100
TGF-β	Transforming growth factor-β
TMB	Tetramethylbenzidine
TNFα	Tumour necrosis factor α
UA PI	Umbilical artery pulsatility index
WHO	World Health Organisation

$N_{glom,kid}$	Total number of PNA-positive glomeruli in the kidney
P_{corp}	Number of renal corpuscles
P_{glom}	Number of glomerular tufts
P_{kid}	Number of grid points falling on kidney tissue
V_{corp}	Mean renal corpuscle volume
$V_{corp,kid}$	Total renal corpuscle volume
V_{glom}	Mean glomerular volume
$V_{glom,kid}$	Total glomerular volume
V_{kid}	Kidney volume

+	plus/positive
-	minus/to/negative
±	plus or minus
x	multiply/times
/	divide/by
=	equal

>	greater than
≥	greater than or equal to
<	less than
≤	less than or equal to
%	percentage
:	ratio
Σ	sum of
²	squared
³	cubed
°	degree
°C	degrees Celcius
~	approximately
α	alpha
β	beta
CI	confidence interval
n	number of experimental animals or litters
r^2	coefficient of determination
rho	Spearman's rank correlation coefficient
P	level of statistical significance
l	litre
ml	millilitre
µl	microlitre
kg	kilogram
g	gram
mg	milligram
µg	microgram
ng	nanogram
cm	centimetre
mm	millimetre
µm	micrometre
nm	nanometre
w/v	weight per volume
w/w	weight per weight
mol	mole
mmol	millimole
MJ	megajoule
kJ	kilojoule
Hz	hertz
min	minute
M	molar
N	normality
pH	power of hydrogen ion
rpm	revolutions per minute
$t_{1/2}$	t-half-life

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Diabetes in pregnancy

Diabetes mellitus is a group of metabolic diseases characterised by hyperglycaemia that arises due to defects in insulin secretion and signalling. Maternal diabetes is a common condition of pregnancy that can have short- and long-term implications for the health and wellbeing of both mother and child. Offspring of diabetic pregnancy are recognised to have an increased risk of congenital malformations, however less overt effects on organ development are not as clearly characterised. The increasing incidence of impaired glucose tolerance in pregnancy calls for a better understanding of the impact of maternal diabetes on fetal development and the consequences this may impose on adult health and disease.

1.1.1 Types of diabetes in pregnancy

Diabetes in pregnancy can be classified into two subtypes: pre-gestational and gestational diabetes.

1.1.1.1 Pre-gestational diabetes

Pre-gestational diabetes may present as either Type 1 or Type 2 diabetes. Type 1 diabetes is typically diagnosed in childhood or early adulthood and results from an absolute deficiency in insulin production and secretion due to the autoimmune destruction of pancreatic β cells (ADA, 2014). Type 1 diabetes affects <1% of pregnancies in Australia (AIHW, 2010). Type 2 diabetes is the more prevalent form of diabetes in the general population and occurs due to insulin resistance and inadequate secretory responses (ADA, 2014). Type 2 diabetes is also strongly associated with obesity. The prevalence of pregnancies complicated by Type 2 diabetes in Australia is approximately 2%, however an additional 14% of women of child-bearing age exhibit impaired glucose tolerance or pre-diabetes signifying a potentially large number of women with undiagnosed glucose intolerance in pregnancy (Dunstan et al., 2002, AIHW, 2008, AIHW, 2010, Razzaghi et al., 2013, CDC, 2014). Given the obesity epidemic and the increasing trends in prevalence of diabetes, the number of women with impaired glucose tolerance present prior to pregnancy and throughout the length of gestation is expected to increase (Bell et al., 2008, Albrecht et al., 2010).

1.1.1.2 Gestational diabetes

Gestational diabetes is defined as any degree of glucose intolerance with onset or first recognition in pregnancy (Hoffman et al., 1998). It is important to note that by definition this may include women with previously undiagnosed pre-gestational diabetes as well as women with true pregnancy-related glucose intolerance. The Australian Institute of Health and Welfare (AIHW) reports gestational diabetes to affect 5% of all pregnancies in Australia, yet depending on the population studied may be as high as 17% (Yue et al., 1996, Shand et al., 2008, Templeton and Pieris-Caldwell, 2008, AIHW, 2010, Moses et al., 2011). Like Australia, the prevalence of gestational diabetes in other Western countries varies

considerably from 2-20%, with 4-6% being the most common rate reported (King, 1998, Hunt and Schuller, 2007, Buckley et al., 2012, Carolan et al., 2012, Jiwani et al., 2012, DeSisto et al., 2014). The frequency of gestational diabetes is reflective of the prevalence of impaired glucose tolerance in women of reproductive age as well as the background prevalence of Type 2 diabetes in that population (Hunt and Schuller, 2007). Variability in the global prevalence of gestational diabetes is therefore related to ethnic and racial differences both between and within countries. Variability in screening approaches and diagnostic criteria used to identify women with gestational diabetes also differs between centres and countries, further contributing to differences in reported prevalence rates and limiting comparability between studies.

Parallel to increases in obesity, Type 2 diabetes and advancing maternal age, the prevalence of gestational diabetes has risen dramatically in recent years. Increased rates of gestational diabetes are reported in Australia (Anna et al., 2008, Abouzeid et al., 2014, Chamberlain et al., 2014) and the Western world (Correa et al., 2014, Feig et al., 2014). Studies reporting the prevalence of gestational diabetes in developing countries are sparse but tend to indicate a high, yet variable, rate of gestational diabetes ranging from 1-28% (Jiwani et al., 2012, Nielsen et al., 2012, Macaulay et al., 2014). The rapid rise in obesity and diabetes in low-income and developing nations due to urbanisation and the adoption of Westernised lifestyles is set to further increase the prevalence of gestational diabetes worldwide.

1.1.1.2.1 Risk factors for gestational diabetes

Women considered at high risk of gestational diabetes may have the following characteristics (AIHW, 2010, Nankervis and Conn, 2013):

- Previous gestational diabetes or elevated blood glucose level
- Ethnicity (South and South-East Asian, Middle Eastern, Indigenous, Pacific Islander and African backgrounds)
- Maternal age ≥ 40 years
- Pre-pregnancy body mass index (BMI) ≥ 30 kg/m^2
- Family history of Type 2 diabetes or gestational diabetes
- Previous macrosomia (baby with birth weight > 4500 g or > 90th percentile)
- Polycystic ovarian syndrome

1.1.1.2.2 Aetiology of gestational diabetes

Pregnancy is associated with an alteration in carbohydrate metabolism to maintain the transfer of nutrients from the maternal to fetal circulation and to ensure the fetus has an adequate supply of glucose (Lain and Catalano, 2007). Due to the presence of circulating placental hormones and

increased fat deposition, pregnancy is accompanied by an increase in insulin resistance from mid-gestation which parallels that observed in individuals with overt Type 2 diabetes. In normal pregnancy, pancreatic β cells compensate by hypertrophy and by altering the balance of proliferation and apoptosis to increase insulin production (Van Assche et al., 1978, Rieck and Kaestner, 2010). The increase in insulin secretion results in relatively small changes in maternal glucose levels throughout pregnancy. If the insulin supply is insufficient to meet tissue demands and regulate maternal blood glucose levels, gestational diabetes can develop.

In a small percentage of cases, women identified with gestational diabetes have autoimmune or monogenic diabetes first detected by routine screening during pregnancy (Catalano et al., 1990). However, the majority of women diagnosed with gestational diabetes exhibit chronic insulin resistance. Pregnancy is associated with a 60% decrease in insulin sensitivity, and is comparable in women with normal glucose tolerance in late gestation and in women diagnosed with gestational diabetes (Catalano et al., 1993). Women with gestational diabetes have been found to exhibit insulin resistance and sub-clinical metabolic dysfunction prior to pregnancy. These underlying defects in insulin sensitivity are unmasked in response to the metabolic stressors of pregnancy (Kim, 2014). Factors contributing to the underlying insulin resistance are poorly defined but may include obesity-related insulin resistance as well as elevated circulating levels of leptin and inflammatory markers (Buchanan and Xiang, 2005, McIntyre et al., 2010).

Compared to women with normal glucose tolerance in pregnancy, women with gestational diabetes also display reduced pancreatic β cell responses given their degree of insulin resistance. The deficit in blood glucose regulation in gestational diabetes arises due to β cell dysfunction characterised as reduced insulin secretion (Homko et al., 2001) and poor first-phase insulin responses (Catalano et al., 1993, Kuhl, 1998, Xiang et al., 1999). Women with gestational diabetes also exhibit a reduction in glucose clearance rates, alterations in insulin signalling pathways and an increase in endogenous glucose production (Ryan et al., 1995, Bowes, 1996, Friedman et al., 1999, Xiang et al., 1999). The biochemical factors and mechanisms associated with β cell dysfunction in gestational diabetes are unclear, but may include altered lipid metabolism and elevated inflammatory cytokines (Catalano, 2014).

1.1.2 Screening and diagnosis of diabetes in pregnancy

Criteria for the testing and diagnosis of diabetes in pregnancy in Australia were formulated over 20 years ago (Martin, 1991). These recommendations were based on expert opinion, had a low evidence base and were designed to identify women at risk for the development of Type 2 diabetes after pregnancy rather than identify those at risk of adverse perinatal and fetal outcomes (Martin, 1991,

IADPSG Consensus Panel, 2010). New consensus guidelines for the testing and diagnosis of gestational diabetes have recently been put forth following the findings of the Hyperglycaemic and Adverse Pregnancy Outcome (HAPO) study (HAPO Study Cooperative Research Group, 2008). The HAPO study was a large, international, multicentre study that investigated fetal and maternal effects of gestational hyperglycaemia in 25,505 women who had a 75 g oral glucose tolerance test (OGTT) between 24-32 weeks gestation. Maternal glycaemia less than overt diabetes (plasma glucose ≤ 5.8 mmol/l fasting and/or ≤ 11.1 mmol/l 2 hours after OGTT) was found to positively correlate with adverse perinatal outcomes including birth weight > 90th percentile, cord C-peptide > 90th percentile (indicative of fetal hyperinsulinaemia), neonatal adiposity > 90th percentile and neonatal hypoglycaemia (HAPO Study Cooperative Research Group, 2008, HAPO Study Cooperative Research Group, 2009). Importantly, there were no obvious thresholds of maternal glycaemia at which risk increased and the associations between maternal glycaemia and perinatal outcomes did not differ across the centres.

Worldwide, a number of screening protocols and diagnostic cut-points have been used to detect glucose abnormalities in pregnancy. The findings of the HAPO study generated an expectation of universal convergence for the adoption of a 75 g OGTT for the diagnosis of gestational diabetes (WHO, 2014). Given the requirement for uniform universal worldwide guidelines for the testing and diagnosis of gestational diabetes, a consensus panel meeting was held in 2008 by the International Association of Diabetes and Pregnancy Study Groups (IADPSG). Based on the findings of the HAPO study and other work identifying an association between maternal glycaemia and short- and long-term health implications for offspring (Sermer et al., 1995, Ferrara et al., 2007, Jensen et al., 2008), IADPSG recommended the diagnostic criteria presented in **Table 1.1** (IADPSG Consensus Panel, 2010). These blood glucose levels represent the odds ratio of 1.75 for the fetal parameters identified in the HAPO study, based on fully adjusted logistic regression models.

Table 1.1 IADPSG guidelines for the diagnosis of gestational diabetes

Glucose test	Fasting plasma glucose	1 hour plasma glucose	2 hour plasma glucose
75 g OGTT* after overnight fasting	≥ 5.1 mmol/l	≥ 10.0 mmol/l	≥ 8.5 mmol/l

*One abnormal glucose value sufficient for diagnosis.

The new recommendations for the testing and diagnosis of gestational diabetes, which include the addition of early pregnancy screening, have been endorsed by many countries and international organisations, including the World Health Organisation (WHO) (WHO, 2014) and the American Diabetes Association (ADA) (ADA, 2011). It should be noted that the ADA now endorses the one-step approach recommended by IADPSG (**Table 1.1**) as well as the traditional two-step approach recommended by the National Institute of Health (NIH) and the American College of Obstetricians and

Gynecologists (ACOG) (ADA, 2014). The two-step strategy involves screening women at 24-28 weeks gestation with a 1 hour 50 g glucose challenge test (GCT) (non-fasting). If glucose levels exceed 7.8mmol/l women proceed to a 3 hour 100 g OGTT. The diagnosis of gestational diabetes is made if two or more glucose values are abnormal (**Table 1.2**).

Despite conflicting guidelines from expert groups within the U.S., there is strong consensus that a uniform universal approach to the testing and diagnosis of gestational diabetes will benefit patients, caregivers and policy makers (ADA, 2014). As glucose intolerance exists on a continuum, defining diagnostic set-points requires balancing the benefits and harms associated with greater or lesser sensitivity. There are currently no available data concerning clinical trial interventions or cost-effective analyses assessing the balance of benefits versus the increased costs of implementing the IADPSG recommendations (ADA, 2014). For these reasons and due to limitations in available infrastructure, universal convergence for the adoption of a 75 g OGTT for the diagnosis of gestational diabetes as recommended by IADPSG has yet to take effect.

Table 1.2 NIH and ACOG guidelines for the diagnosis of gestational diabetes

Glucose test	Diagnostic criteria	
STEP 1: 50 g 1 hour GCT (non-fasting)	If 1 hour glucose ≥ 7.8 mmol/l, proceed to STEP 2	
STEP 2: 100 g 3 hour OGTT after overnight fasting	**Carpenter/Coustan***	**National Diabetes Data Group***
Fasting plasma glucose	≥ 5.3 mmol/l	≥ 5.8 mmol/l
1 hour plasma glucose	≥ 10.0 mmol/l	≥ 10.6 mmol/l
2 hour plasma glucose	≥ 8.6 mmol/l	≥ 9.2 mmol/l
3 hour plasma glucose	≥ 7.8 mmol/l	≥ 8.0 mmol/l

*Two or more abnormal glucose values at OGTT required for diagnosis.

Within Australia, the Australasian Diabetes in Pregnancy Society (ADIPS) (ADIPS, 2014), the Royal Australasian College of Obstetrics and Gynaecology (RANZCOG) and the Royal College of Pathologists Australia (RCPA) have endorsed the recommendations of IADPSG. Note that WHO and ADIPS have fully endorsed the IADPSG recommendations, with the exception of referring to women with a diabetic result early in pregnancy as having 'overt diabetes'. These women are instead referred to as having 'diabetes mellitus in pregnancy', with the matter to be resolved in the post-partum period.

Previous guidelines for the testing and diagnosis of gestational diabetes within Australia incorporated screening women at 24-28 weeks gestation with a 1 hour 50 g GCT (non-fasting) who would then proceed to a 2 hour 75 g OGTT if abnormal. The GCT lacks both sensitivity and specificity and is no longer used in the diagnosis of gestational diabetes in Australia. Recommendations for routine testing and diagnosis of gestational diabetes in Australia are presented in detail below (ADIPS, 2014).

Early screening for diabetes in pregnancy at first antenatal booking:

Women deemed at higher risk of hyperglycaemia in pregnancy (**Section 1.1.1.2.1)** should have a 75 g OGTT or HbA1c (glycosylated haemoglobin) measured early in pregnancy, and if abnormal may be diagnosed with diabetes mellitus in pregnancy or gestational diabetes. All other women should have fasting or random plasma glucose levels measured at first booking.

Routine testing for gestational diabetes at 24-28 weeks:

All women not diagnosed with diabetes mellitus in pregnancy or gestational diabetes at first antenatal booking should have a 75 g OGTT at 24-28 weeks gestation.

Diagnostic criteria for hyperglycaemia at any time in pregnancy:

Diabetes mellitus in pregnancy:

- Fasting plasma glucose level ≥ 7.0 mmol/l
- 2 hour plasma glucose level ≥ 11.1 mmol/l following 75 g oral glucose load
- Random plasma glucose level ≥ 11.1 mmol/l in the presence of diabetes symptoms
- HbA1c ≥ 48 mmol/mol

*One abnormal glucose value sufficient for diagnosis

Gestational diabetes:

- Fasting plasma glucose level 5.1 - 6.9 mmol/l
- 1 hour plasma glucose level ≥ 10.0 mmol/l*
- 2 hour plasma glucose level ≥ 8.5 mmo/l

*Note: there are no established criteria for the diagnosis of diabetes mellitus in pregnancy based on the 1-h post-load value.

*One abnormal glucose value sufficient for diagnosis

It should be noted that there is only limited evidence for the use of HbA1c in diagnosis, management and post-partum assessment of women with gestational diabetes. Evidence is also limited regarding whether early testing for gestational diabetes improves pregnancy outcomes. Further studies are required to address whether early detection and diagnosis with HbA1c improve pregnancy outcomes.

Previous and current criteria for the diagnosis of gestational diabetes in Australia are presented in **Table 1.3**. Based on the new international diagnostic criteria, the prevalence of gestational diabetes in Australia is expected to increase to 12-14%. The total incidence of gestational diabetes in the HAPO cohort after applying the IADPSG criteria was 17.8% (IADPSG Consensus Panel, 2010), with the two Australian sites demonstrating a prevalence of 12.1% (Brisbane) and 13.6% (Newcastle), with the

majority of women diagnosed by a fasting glucose measure (Sacks et al., 2012). A prospective study in Wollongong reported an increase in prevalence from 9.6% to 13% between the old and new recommendations (Moses et al., 2011). Compared to previous criteria, the IADPSG criteria include a lower threshold for fasting glucose, a higher 2-hour glucose threshold and a new 1-hour glucose threshold value. The number of women identified with gestational diabetes will likely vary between population groups, due to differences in fasting hyperglycaemia and glucose tolerance between different ethnicities (McIntyre et al., 2015).

Table 1.3 Previous and current ADIPS criteria for the diagnosis of gestational diabetes

	Fasting	1 hour	2 hour
Previous ADIPS criteria	≥ 5.5 mmol/l	N/A	≥ 8.0 mmol/l
IADPSG and new ADIPS criteria	≥ 5.1 mmol/l	≥ 10.0 mmol/l	≥ 8.5 mmol/l

*Based on 75 g OGTT at 24-28 weeks

*One abnormal glucose value sufficient for diagnosis

1.1.3 Treatment of diabetes in pregnancy

Strict control of blood glucose levels should be pursued in all women with diabetes in pregnancy, and women should be managed by a multidisciplinary team (McElduff et al., 2005a, Ballas et al., 2012).

Consensus guidelines for the management of pregnancy in women with Type 1 or Type 2 diabetes have been published by ADIPS (McElduff et al., 2005a). Women with pre-existing Type 1 diabetes are advised to establish an insulin treatment plan throughout pregnancy. Up to mid-gestation these women are prone to hypoglycaemic periods and insulin doses may need to be reduced (McElduff et al., 2005b). However as the physiological insulin resistant state of pregnancy increases, the requirements for insulin may also increase considerably. Some women with Type 1 diabetes may benefit from an insulin pump during pregnancy (Gabbe et al., 2000). HbA1c levels, as a marker of long-term glycaemic control, should be monitored regularly and be maintained within the reference range (<53mmol/mol). Women are also advised to undergo formal assessment for diabetes complications such as proteinuria and retinopathy and should be monitored regularly. Women with pre-existing Type 2 diabetes may be managed with diet and exercise but insulin treatment is typically required in late gestation (McElduff et al., 2005b). Treatment targets and screening for complications in pregnancy in women with Type 2 diabetes are as for women with Type 1 diabetes. The following blood glucose targets are recommended for women with pre-gestational diabetes (McElduff et al., 2005a):

- Fasting blood glucose: 4.0-5.5 mmol/l
- 1 hour postprandial blood glucose ≤ 8.0 mmol/l
- 2 hour postprandial blood glucose ≤ 7.0 mmol/l

Many women diagnosed with gestational diabetes can be primarily managed with dietary therapy and moderate physical exercise, however in some cases insulin treatment is required to achieve euglycaemia. The type and dose of insulin needed to control glucose levels depends on the severity and pattern of hyperglycaemia, and also tends to increase as pregnancy progresses (Jovanovic, 2004). The following self-monitored treatment targets are recommended for women with gestational diabetes (ADIPS, 2014):

- Fasting blood glucose ≤ 5.0 mmol/l
- 1 hour postprandial blood glucose ≤ 7.4 mmol/l
- 2 hour postprandial blood glucose ≤ 6.7 mmol/l

Intervention studies demonstrate the benefit of treating gestational diabetes. Two randomised clinical trials have reported a reduction in serious perinatal complications, birth trauma, birth weight and incidence of fetal overgrowth in women treated with dietary advice, blood glucose monitoring and insulin therapy if necessary, compared to routine care (Crowther et al., 2005, Landon et al., 2009).

Women diagnosed with diabetes during pregnancy are at risk of developing diabetes after birth as glucose intolerance and reduced insulin secretion can persist. A systematic review by Kim et al. (2002) found a cumulative incidence of Type 2 diabetes of up to 70% in a 10 year post-partum period, and a systematic review by Bellamy et al. (2009) found women with gestational diabetes had a 7-fold increased risk of developing Type 2 diabetes compared with women who had a normoglycaemic pregnancy. These women are also at an increased risk of metabolic syndrome and vascular disease (Kaaja and Ronnemaa, 2008, Xu et al., 2014). In the post-partum period, women are advised to continue monitoring glucose and are recommended to have a 75 g OGTT at 6-12 weeks post-partum and every 1-2 years thereafter (ADIPS, 2014).

1.2 The effects of diabetes in pregnancy on offspring

1.2.1 Congenital anomalies

It is well recognised that infants of women with Type 1 diabetes have an increased rate of congenital abnormalities (Molsted Pedersen et al., 1964, Kucera, 1971). Termed fuel-mediated teratogenesis or diabetic embryopathy, offspring of women with Type 1 diabetes have a 2-5 fold greater incidence of congenital anomalies than offspring of women without diabetes (Nielsen et al., 2005). The risk of congenital anomalies is related to the standard of care and glycaemic control: malformation rates are lower in women with pre-conception care than women with post-conception care (Kitzmiller et al., 1991) and are higher in women with poorly controlled diabetes in early pregnancy (Miller et al., 1981).

The structural anomalies typically occur within the first 8 weeks of gestation during the period of organogenesis, before many diabetic pregnant women attend antenatal medical appointments.

Malformations reported in infants of women with Type 1 diabetes predominantly affect the cardiovascular system, central nervous system, skeletal system as well as kidneys and urinary tract (Becerra et al., 1990, Martinez-Frias, 1994, Nold and Georgieff, 2004). Common neurological anomalies include anencephaly, hydrocephaly, spina bifida and encephalocaele. Common cardiovascular anomalies include transposition of the great vessels, ventricular septal defects and patent ductus arteriosus. Specific anomalies of the kidney and urinary tract include renal agenesis, renal dysgenesis, obstructive defects of the urinary tract and hydronephrosis. An increased incidence of lower limb deficiency and spinal anomalies are also reported, as is caudal regression syndrome, or sacral agenesis, a well-established congenital abnormality of diabetic pregnancy (Bánhidy et al., 2010). Infants of women with Type 1 diabetes are also at increased risk of multiple congenital anomalies (Martinez-Frias, 1994, Bánhidy et al., 2010).

Studies have reported a 2-4 fold increase in the incidence of congenital malformations in infants of women with pre-gestational diabetes where data on women with Type 1 and Type 2 diabetes are combined (Aberg et al., 2001, Sheffield et al., 2002, Nielsen et al., 2005, Sharpe et al., 2005). Studies addressing the rate of congenital anomalies in women with Type 2 diabetes alone are less numerous but do report an increased rate of congenital malformations, comparable to that reported in women with Type 1 diabetes (Towner et al., 1995, Farrell et al., 2002, Macintosh et al., 2006, Murphy et al., 2011). Findings are however inconsistent, as Bánhidy et al. (2010) and Martinez-Frias (1994) reported no increased risk of total congenital anomalies in infants of Type 2 diabetes compared with control pregnancies, yet did show greater occurrence of specific defects including diaphragmatic anomalies and spinal anomalies. Conversely, increased rates of congenital malformations in Type 2 diabetes, above that of Type 1 diabetes, are also reported (Roland et al., 2005). Women with Type 2 diabetes have greater BMI and lower rates of pre-pregnancy counselling which may contribute to this variability (Roland et al., 2005, Murphy et al., 2011)

Congenital abnormalities in offspring of women with gestational diabetes have also been reported, yet to a lesser and more variable extent than for Type 1 diabetes. Several studies report normal total malformation rates in gestational diabetes (Aberg et al., 2001, Farrell et al., 2002, Bánhidy et al., 2010), although Sharpe et al. (2005) and Feig et al. (2014) reported slightly greater total malformation rates (18% and 26% increased risk, respectively) yet did not adjust for maternal BMI. Other studies show a greater incidence of specific anomalies. Aberg et al. (2001) found a greater incidence of cardiac defects, intestinal atresia and spinal malformations whereas Martínez-Frías et al. (1998) found an increased

rate of holoprosencephaly, spinal defects, and renal and urinary tract defects. These findings indicate that a sub-group of women diagnosed with gestational diabetes are at an increased risk of congenital malformations, comparable to rates reported in women with Type 1 diabetes (Aberg et al., 2001). This may be related to insulin requirements as Becerra et al. (1990) found a greater rate of cardiac defects only in gestational diabetic women treated with insulin. Increased malformation rates have also been identified in women with fasting hyperglycaemia, but not in women with normal fasting glucose levels (Schaefer et al., 1997, Schaefer-Graf et al., 2000, Sheffield et al., 2002). Furthermore, the sub-group of women with gestational diabetes at greatest risk of congenital malformations may be those with undiagnosed pre-existing Type 2 diabetes, that can only be confirmed post-partum, or may be related to higher BMI (Farrell et al., 2002).

Infants of diabetic mothers are prone to a range of structural defects encompassing a number of organ systems, indicating a non-specific effect of hyperglycaemia on fetal development. The aetiology of congenital abnormalities in diabetic pregnancy is unclear but may be related to the accumulation of toxic metabolites and nutrient deficiencies in the presence of hyperglycaemia (Allen et al., 2007). Excess reactive oxygen species (ROS) have been implicated in fuel-mediated teratogenesis. Fetal hyperglycaemia and excessive glucose metabolism can lead to an increase in the production of ROS, and due to the relative immaturity of the fetal free radical scavenging system, can lead to oxidative stress. This in turn may lead to widespread cellular and embryonic damage due to protein glycosylation, lipid peroxidation, altered molecular signalling pathways, an increase in apoptotic events and DNA damage (Ornoy, 2007, Eriksson, 2009). Embryopathies may also be related to a deficiency in specific plasma membrane lipids including arachidonic acid and myoinositol, leading to disruption of intracellular signalling in the hyperglycaemic embryo. Experimental administration of antioxidants, arachidonic acid and myoinositol have been shown to reduce the rate of embryonic malformations in *in vivo* rodent models of diabetic embryopathy and may have therapeutic potential in the clinical setting (Reece and Homko, 2000). This is important given that the risk of congenital malformations in women with diabetes, especially pre-gestational diabetes, is still greater than the general population despite increased efforts to improve glycaemic control in diabetic women.

1.2.2 Adverse perinatal outcomes

In addition to an increased rate of congenital malformations, pregnancies complicated by diabetes are at an increased risk of adverse perinatal outcomes. Pregnancies complicated by Type 1 diabetes are at greater risk of still-birth and perinatal mortality, pre-eclampsia, pre-term delivery, induced labour, instrumental delivery, Caesarean section and fetal distress (Persson et al., 2009, AIHW, 2010, Colstrup et al., 2013). Infants of women with Type 1 diabetes are also likely to be born at lower gestational age but with higher birth weight and at greater risk of neonatal morbidities including lower Apgar scores

(summarises the health of a newborn child and is based on Appearance, Pulse, Grimace, Activity and Respiration) and increased rates of admission to neonatal intensive care units (Jensen et al., 2004).

Infant birth weight is an important measure of health in the newborn, and both low and high birth weight are associated with increased morbidity and mortality. High birth weight is a common outcome of diabetic pregnancy and can be defined as macrosomia (birth weight > 4000g or > 4500g, variable between studies) or as large for gestational age (LGA; birth weight > 90th percentile). Fetal overgrowth occurs in diabetic pregnancy due to fetal hyperglycaemia and hyperinsulinaemia, as postulated by Pedersen (1954). Because glucose but not insulin readily crosses the placenta, fetal hyperglycaemia results in fetal pancreatic islet cell hypertrophy and increased fetal insulin production. As insulin is a major anabolic hormone and the main fetal growth factor, hyperinsulinaemia leads to an increase in fat and protein stores, adiposity and accelerated fetal growth. Fetal weight accretion typically occurs from 32 weeks gestation, and may be associated with greater abdominal circumference in relation to head circumference, as well as hepatomegaly, splenomegaly and cardiomegaly (Nold and Georgieff, 2004, Negrato et al., 2012). Despite improvements in the diagnosis and treatment of women with Type 1 diabetes, high birth weight is still a common outcome with over 50% of infants born LGA and over 20% born macrosomic – rates that are 2-5 fold higher than in infants born to non-diabetic mothers (Klemetti et al., 2012, Colstrup et al., 2013, Gizzo et al., 2013).

Not surprisingly, fetal macrosomia detected by obstetric ultrasound contributes to the higher rate of Caesarean delivery, labour induction, and pre-term delivery reported in diabetic pregnancies (Persson et al., 2009). However, spontaneous pre-term birth is also increased in Type 1 diabetic pregnancies (Evers et al., 2004). Macrosomia at birth is a marker for subsequent neonatal morbidity in infants of Type 1 diabetic pregnancy. Fetal overgrowth in diabetic pregnancy increases the risk of perinatal and neonatal complications including shoulder dystocia, birth trauma, neonatal hypoglycaemia, respiratory distress syndrome, hyperbilirubinaemia, and polycythaemia (Negrato et al., 2012). Neonatal hypoglycaemia is seen in up to 50% of newborns of diabetic pregnancy (Evers et al., 2004) and occurs due to the combination of neonatal hyperinsulinaemia and the sudden interruption of glucose delivery in the post-partum period. Fetal hyperinsulinaemia can also delay lung maturation and surfactant production, leading to respiratory distress syndrome and transient tachypnoea (retained fetal lung fluid) and an increased requirement for high-level resuscitation (Persson et al., 2009). Cardiac intraventricular septal hypertrophy is also reported in infants of diabetic pregnancy due to increased glycogen loading. Fetal hyperglycaemia and hyperinsulinaemia can also stimulate erythropoiesis and haemolysis, giving rise to hyperbilirubinaemia and polycythaemia in the newborn. Neonatal morbidities (one or more complications) have been reported in between 50-80% of Type 1 diabetic pregnancies (Evers et al., 2004, Owens et al., 2015).

It is important to note that a small subgroup of women with pre-existing diabetes (2-10%) (Nold and Georgieff, 2004, McIntyre et al., 2009) may have infants with low birth weight, defined as birth weight <2500g or as small for gestational age (SGA; birth weight <10th percentile). Fetal growth deceleration is typically seen in women with advanced diabetic vascular disease, hypertension and placental vascular insufficiency leading to fetal malnutrition and hypoxia (Howarth et al., 2007, Glinianaia et al., 2012). Low birth weight due to maternal diabetes is associated with adverse perinatal outcomes including perinatal mortality, preterm delivery and neonatal hypoglycaemia.

Similar adverse perinatal outcomes are reported in women with Type 2 diabetes. Compared to women with Type 1 diabetes, women with Type 2 diabetes are typically older, have a higher BMI, are of non-Caucasian ethnicity, have a shorter disease duration and generally have lower mean HbA1c values across pregnancy (Hillman et al., 2006, Murphy et al., 2011, Gizzo et al., 2013, Owens et al., 2015). Despite these parameters, women with Type 2 diabetes present with similar rates of adverse perinatal outcomes to those of Type 1 diabetes including pre-term delivery, Caesarean section, macrosomia and neonatal morbidities (Roland et al., 2005, Lapolla et al., 2008, Gizzo et al., 2013, Owens et al., 2015). While some studies report fewer neonatal morbidities in infants of Type 2 diabetic women (Murphy et al., 2011, Owens et al., 2015), there is a growing body of evidence indicating that certain outcomes may be worse in Type 2 diabetes compared to Type 1 diabetes, with higher rates of still-birth, perinatal mortality and SGA infants reported (Clausen et al., 2005, Roland et al., 2005, Cundy et al., 2007, AIHW, 2010, Murphy et al., 2011, Gizzo et al., 2013). This may be explained by the presence of metabolic syndrome, obesity and inflammation in women with Type 2 diabetes, although significantly worse obstetric and maternal/fetal outcomes are reported in women with Type 2 diabetes compared to non-diabetic women when matched for BMI (Knight et al., 2012).

Gestational diabetes is also associated with an increased rate of adverse perinatal outcomes, yet to a lesser extent than pre-gestational diabetes (Shand et al., 2008). Compared to non-diabetic pregnancies, gestational diabetic pregnancies are associated with a greater risk of Caesarean delivery, shoulder dystocia, neonatal intensive care unit admission, pre-term delivery, LGA infants, macrosomia, neonatal adiposity, respiratory distress, and neonatal hyperinsulinaemia, hypoglycaemia and hyperbilirubinaemia (Shand et al., 2008, Esakoff et al., 2009, Catalano et al., 2012). A 2-3 fold increased risk of high birth weight is reported in gestational diabetes, putting infants at greater risk of the adverse perinatal outcomes seen in pre-gestational diabetes (Persson et al., 2013). Several studies indicate that maternal glucose intolerance and hyperglycaemia below the threshold for the diagnosis of gestational diabetes are associated with neonatal morbidities. Linear trends are reported between maternal glycaemia within a non-diabetic range and Caesarean section, pre-term delivery, LGA infants, hyperinsulinaemia, neonatal hypoglycaemia and shoulder dystocia, with no obvious threshold at which

the risk of adverse perinatal outcomes increase (Sermer et al., 1995, HAPO Study Cooperative Research Group, 2008, Jensen et al., 2008). Similar associations between maternal glycaemic control and neonatal morbidity are identified in pre-gestational Type 1 and Type 2 diabetes: increasing third trimester HbA1c values predict higher birth weight (Glinianaia et al., 2012) as well as neonatal macrosomia, low APGAR score, neonatal intensive care admission and neonatal hypoglycaemia (Klemetti et al., 2012).

1.2.3 Developmental programming

The concept that an altered intrauterine environment can exert permanent effects on metabolic function is termed developmental programming or the 'developmental origins of health and adult disease' hypothesis (Langley-Evans and McMullen, 2010). This hypothesis was originally based on studies by Barker and Osmond (1986) who found associations between low birth weight and cardiovascular disease, hypertension and metabolic syndrome in adulthood (Barker et al., 1993). Since then, an extensive body of evidence has emerged to support the concept that an adverse perinatal environment leading to fetal undernutrition can increase predisposition to adult disease. Moreover, it is apparent that excessive fetal growth and fetal overnutrition, as seen in diabetic pregnancy, can also program metabolic dysfunction in adulthood. Fuel mediated teratogenesis can be viewed in terms of congenital malformations arising due to excessive nutrient supply in early development and organogenesis. As an extension of this, more subtle forms of teratogenesis may also arise following prolonged exposure to altered maternal metabolism and hyperglycaemia into the neonatal period, which may have long-range effects in the offspring, as postulated by Freinkel (1980). Numerous prospective cohort studies have since demonstrated metabolic dysfunction among offspring exposed to maternal diabetes.

1.2.3.1 Intrauterine hyperglycaemia is associated with long-term metabolic consequences

The effect of the fetal environment and intrauterine hyperglycaemia on subsequent adult disease was first elucidated by Doerner (1975) who observed an aggregation of familial diabetes on the maternal side rather than the paternal side in subjects with diabetes. The effect of maternal diabetes on offspring health has largely been demonstrated in Pima Indians, a Native American population in Arizona who have the highest prevalence and incidence of Type 2 diabetes in the world (Bennett et al., 1971). Interestingly, Pima Indian populations in Mexico with considerable genetic similarity do not exhibit this high prevalence, highlighting the role of the environment and transition to a Westernised lifestyle in the development of glucose intolerance (Schulz et al., 2006). In the Pima Indian population of Arizona, offspring of women with Type 2 diabetes have a significantly elevated prevalence and earlier diagnosis of Type 2 diabetes, obesity and hypertension above that of offspring of non-diabetic

women and above genetic susceptibility (Pettitt et al., 1983, Pettitt et al., 1988, Charles et al., 1994, Dabelea et al., 2000, Bunt et al., 2005). Furthermore, both normal and high birth weight offspring of Type 2 diabetic pregnancy have an increased risk of glucose intolerance and obesity.

Studies in multiethnic populations have reported comparable results: exposure to maternal diabetes (combined pre-gestational and gestational diabetes) is associated with a 3-5 fold increased risk of obesity, impaired glucose tolerance and Type 2 diabetes in adolescents; outcomes that correlate with fetal hyperinsulinaemia (Silverman et al., 1995, Silverman et al., 1998, Dabelea et al., 2008, Pettitt et al., 2008). Increased BMI and systolic blood pressure have also been reported in a multi-ethnic cohort of children exposed to maternal diabetes (Crume et al., 2011, West et al., 2011), highlighting poorer profiles of cardiovascular and diabetic risk in early life. Unfortunately, studies investigating long-term outcomes in offspring of women without Type 2 diabetes outside of the Pima Indian community are sparse.

A number of epidemiological studies have assessed the effect of Type 1 diabetes or gestational diabetes on offspring health and wellbeing. Offspring of women with pre-gestational Type 1 diabetes have a 2-4 fold greater risk of pre-diabetes, Type 2 diabetes, overweight and metabolic syndrome, including increased systolic blood pressure in adulthood (Weiss et al., 2000, Sobngwi et al., 2003, Clausen et al., 2008, Clausen et al., 2009, Vlachova et al., 2015). Similar outcomes of metabolic dysfunction are reported in offspring of gestational diabetes, with some reports highlighting worse outcomes in offspring exposed to gestational diabetes than Type 1 diabetes (Boney et al., 2005, Clausen et al., 2008, Clausen et al., 2009, Järvelin et al., 2009, Wright et al., 2009). Nonetheless, together these studies highlight that exposure to hyperglycaemia itself exerts a detrimental long-term effect on the fetus, irrespective of the aetiology of maternal diabetes. In the coming years, data from the HAPO-Follow-up Study, which aims to assess maternal and child health in 7000 participants of the original HAPO study, will provide further information on levels of maternal hyperglycaemia in pregnancy and the risk of childhood obesity and glucose metabolism.

1.2.3.2 Mechanisms of developmental programming

It is well recognised that offspring of diabetic pregnancy have an increased risk of metabolic dysfunction in later life, however the mechanisms leading to these effects remain unclear. Programming of abnormal glucose tolerance in intrauterine hyperglycaemia may occur due to epigenetic processes altering gene expression and genetic imprinting, oxidative stress imposed by elevated glucose in the fetal compartment, and hyperinsulinaemia. Animal studies show that exposure to high glucose during a critical window of development can lead to altered morphology of the pancreas (Aerts et al., 1990) affecting organ and islet cell mass, altered angiogenesis and perturbed

insulin signalling and hypothalamic pathways of energy balance (Plagemann et al., 1999). These mechanisms are reviewed in detail by Poston et al. (2011) and Fetita et al. (2006).

1.2.3.3 The transmission of diabetes to subsequent generations

The multitude of human studies that have reported an increased risk of glucose intolerance and obesity in offspring of diabetic women, who are themselves commonly overweight and glucose intolerant, highlights how these metabolic alterations can be transmitted from one generation to the next. Consequently, these metabolic abnormalities are prevalent in women of child bearing age and is therefore part of a vicious cycle that increases the prevalence of diabetes and other metabolic disorders in subsequent generations (Dabelea et al., 2000, Alcolado et al., 2002).

1.3 Obesity in pregnancy

Obesity is a global epidemic that is attributed to physical inactivity, urbanisation, and the consumption of highly pleasurable, energy dense foods, resulting in an imbalance in the number of calories consumed and expended. The prevalence of obesity has doubled in the past 20 years in Australia, the United States, and other Western countries (Main et al., 2010, Walls et al., 2010). Developing nations also demonstrate increased obesity rates (Popkin et al., 2012). In Australia, 35% of women aged 25-35 years are overweight (BMI ≥ 25kg/m^2 and < 30kg/m^2) or obese (BMI ≥ 30kg/m^2) (Cameron et al., 2003) and it is estimated that 50% of women enter pregnancy either overweight or obese (Athukorala et al., 2010, McIntyre et al., 2012, ACOG, 2013). Obesity is the strongest risk factor for the development of glucose intolerance in pregnancy as well as the development of Type 2 diabetes in non-pregnant individuals (Catalano, 2010). Almost half of all women diagnosed with gestational diabetes are overweight, obese or extremely obese (Kim et al., 2010). The majority of women with Type 2 diabetes in pregnancy also have a BMI greater than 25 kg/m^2 (AIHW, 2008). Women with Type 1 diabetes have also not escaped the obesity epidemic (Klemetti et al., 2012). The strong association between BMI and glucose intolerance can be attributed to decreased insulin sensitivity and insulin secretion and altered lipid metabolism and inflammatory responses (Buchanan and Xiang, 2005, Catalano, 2010). Given the association between maternal obesity and diabetes, it is important to consider the potentially confounding effect of obesity when assessing the role of maternal diabetes on offspring development and health, and vice versa.

Obesity is a recognised risk factor for congenital malformations and adverse perinatal outcomes (Sebire et al., 2001, Stothard et al., 2009, Avci et al., 2014), however few studies control for maternal glycaemia. Those that do adjust for pre-existing diabetes and glucose intolerance in pregnancy report a 2-fold greater risk of total congenital malformations in obese but not overweight women compared to women of recommended bodyweight (Callaway et al., 2006, Owens et al., 2010), with greater rates

of cardiac defects and spina bifida (Cedergren and Kallen, 2003, Waller et al., 2007, Brite et al., 2014, Cai et al., 2014). Overweight and obese women with normal glucose tolerance have a greater risk of Caesarean section, hypertensive disorders of pregnancy, LGA infants, neonatal hyperinsulinaemia, neonatal adiposity and admission to neonatal intensive care units than women with normal body weight and normal glucose tolerance (Callaway et al., 2006, HAPO Study Cooperative Research Group, 2010, Owens et al., 2010, Catalano et al., 2012, Gilead et al., 2012).

Several studies have assessed the relative contributions of hyperglycaemia and obesity on obstetric and neonatal outcomes. As part of the HAPO study, Catalano et al. (2012) reported comparably higher rates of adverse perinatal outcomes in women with untreated gestational diabetes or obesity compared to women with normal glucose tolerance and BMI. In a U.S. population, (Langer, 2015) also found similarly increased rates of neonatal morbidities between women with untreated gestational diabetes or obesity, although gestational diabetes was associated with a greater risk of Caesarean delivery and labour induction. In both of these studies the combination of gestational diabetes and obesity had a greater impact than either variable alone. Moreover, progressively greater odds of LGA infants were reported with both increasing maternal BMI and glucose intolerance (Catalano et al., 2012, Langer, 2015). While the rates of adverse perinatal outcomes reported in women with either obesity or hyperglycaemia are generally comparable, maternal BMI has a much stronger population impact than maternal glucose intolerance due to the greater prevalence of obesity in pregnancy (Ehrenberg et al., 2004, Ricart et al., 2005).

The mechanisms by which maternal obesity leads to congenital defects and adverse perinatal outcomes are not clearly defined. Maternal hyperinsulinaemia, low serum folate levels and deficiencies in certain micronutrients due to poor quality diets may contribute to the greater incidence of congenital defects in obese women (Rasmussen et al., 2008). The increased rate of adverse perinatal outcomes may be related to maternal dyslipidaemia and a greater supply of triglycerides, FFAs and amino acids to the fetal compartment. In turn, this may promote fetal hyperinsulinaemia and increase the risk of other neonatal morbidities, as seen in pregnancies complicated by hyperglycaemia (Schaefer-Graf et al., 2008, HAPO Study Cooperative Research Group, 2010). Obesity is also associated with chronic inflammation, which may further contribute to adverse maternal and neonatal outcomes (Ramsay et al., 2002).

1.4 The role of the placenta

The placenta is a complex organ that provides a selective barrier between the maternal and fetal circulations. The anatomical configuration of the placenta prevents the direct contact of maternal and fetal blood and therefore has an important role in gas exchange and the regulation of nutrient

transport. Fetal growth is reliant on the availability of nutrients in the maternal circulation and the transport of nutrients into the fetal circulation via the placenta. Pathologies of pregnancy may alter placental morphology, growth and function, which in turn may impact growth and development of the fetus.

In the human placenta, gases, water and substrates pass through two cell layers to reach the fetus – the epithelial syncytiotrophoblast and the fetal capillary endothelium. Cells of the syncytiotrophoblast have two polarised membranes: the apical microvillous membrane that is bathed in maternal blood in the intervillous space; and the basal plasma membrane that is directed towards fetal capillaries (Brett et al., 2014). The transfer of substrates from the maternal to fetal circulation is dependent on uteroplacental and umbilical blood flow, available exchange area, placental metabolism, concentration gradients and the expression and activity of nutrient-specific membrane-bound transporters of the syncytiotrophoblast (Gaccioli et al., 2013a).

1.4.1 Placental transport of macronutrients

Glucose, amino acids and free fatty acids (FFAs) are essential macronutrients for adequate fetal growth and development. Glucose is the major energy source for the fetus and is required for fetal growth (Lager and Powell, 2012). Glucose transport is mediated via facilitated diffusion along a concentration gradient, and thus maternal hyperglycaemia drives the net flux of glucose towards the fetus. Glucose is predominantly transported across the microvillous membrane by glucose transporter 1 (GLUT1), and to a lesser extent by GLUT3 (Illsley, 2000).

Amino acids are the building blocks of proteins and are essential for fetal development. Plasma concentrations of amino acids are generally higher in the fetal circulation than the maternal circulation, reflecting active transport across the syncytiotrophoblast (Cetin et al., 1992). The human placenta expresses more than 20 amino acid transporters in the syncytiotrophoblast (Jansson, 2001). The two most prevalent amino acid transport systems are System A (a sodium-dependent accumulative transport system which facilitates the transport of small neutral amino acids) and System L (a sodium-independent exchanger for large neutral amino acids, whereby non-essential amino acids are exchanged for essential amino acids).

Fatty acids are an energy source, are a structural component of cellular membranes and are required for brain development and fat accretion in the fetus. FFAs are readily transported across the placenta, and thus fetal lipid profiles generally reflect that of the maternal circulation (Haggarty, 2010). Placental uptake of non-esterified fatty acids may occur by simple diffusion, or selective transport. Lipoprotein lipase and endothelial lipase are located on the microvillous membrane of the placenta, and hydrolyse maternal triglycerides to FFAs. FFAs can then cross the microvillous membrane via fatty acid transport

proteins (FATP), fatty acid translocase (FAT) and through syncytiotrophoblast cytosol by fatty acid binding proteins (FABP) (Lager and Powell, 2012).

1.4.2 Altered placental nutrient transport in maternal diabetes and obesity

Diabetic and obese women typically have elevated serum levels of hormones such as insulin, leptin and insulin like growth factor I (IGF-I), and cytokines such as interleukin-6 (IL6) and tumour necrosis factor α (TNFα). These factors have been shown to stimulate placental glucose, amino acid and FFA transporters and have been proposed to promote fetal growth (Jones et al., 2007, Gaccioli et al., 2013a). Specific alterations in placental function and nutrient transport are associated with pathological fetal growth: the activity and expression of System A and System L amino acid transporters and ion transporters are reduced in intrauterine growth restriction, whereas the activity and expression of System A, System L and GLUT are increased in accelerated fetal growth (Jansson and Powell, 2006, Brett et al., 2014). The placenta can therefore be considered a nutrient sensor, altering placental transport and nutrient availability to the fetus according to maternal supply and hormonal regulation.

Compared to non-diabetic pregnancy, Type 1 diabetes is associated with an increase in GLUT1 expression and glucose uptake at the basal plasma membrane, an increase in lipoprotein lipase activity, and an increase in the expression of FABPs and endothelial lipase (Jansson et al., 1999, Magnusson et al., 2004, Lindegaard et al., 2006, Radaelli et al., 2009). Alterations in placental nutrient transport are associated with fetal overgrowth and macrosomia, even in women with apparent optimal glycaemic control. Increased System A activity has also been reported in Type 1 diabetic pregnancies independent of fetal growth (Jansson et al., 2002). Together these studies demonstrate that pre-gestational Type 1 diabetes can alter placental nutrient transport, in turn altering fetal nutrition and growth trajectories.

Findings from studies of placental nutrient transport in gestational diabetes are more variable. Fetal overgrowth in gestational diabetes is associated with upregulated FABPs protein expression, System A activity and leucine transport (Jansson et al., 2002, Magnusson et al., 2004, Radaelli et al., 2009), however reports of expression profiles of microvillous GLUTs and lipases are inconsistent. The discrepancy reported between studies (as reviewed by Brett et al. (2014) and Jansson and Powell (2006)) is likely multifactorial and may be related to differences in study populations regarding insulin treatment and maternal, placental and fetal weights, as well as differences in methodologies used to assess transporter expression and activity.

Few studies have assessed placental nutrient transport in obesity. Only one study has examined the effect of maternal obesity on placental glucose transporters, wherein no difference in GLUT1

expression was reported (Colomiere et al., 2009). Amino acid transporters, specifically System A but not System L, are increased in obese women with LGA infants (Jansson et al., 2013). Upregulated expression of FAT, FABPs, endothelial lipase and lipoprotein lipase are also reported in obese women indicating an increased capacity to supply lipids to the fetus (Gauster et al., 2011, Scifres et al., 2011, Dube et al., 2012). Other studies report no difference, or even decreased amino acid and fatty acid placental transport in obesity (Brett et al., 2014). Inconsistences in the direction of change may be related to differences in study populations, or may reflect compensatory changes made by the fetus to regulate nutrient supply in maternal excess. Further studies assessing placental nutrient transport and function and their association with perinatal outcomes in gestational diabetic and obese pregnancies are required.

1.4.3 Other placental alterations in diabetic and obese pregnancy

Asides from specific changes in placental nutrient transporters, women with diabetes exhibit a greater placental exchange surface area, a thickening of the trophoblastic basement membrane, increased villous inflammatory cells and cytokine release, as well as increased placental vascularity (Teasdale and Jean-Jacques, 1986, Sutton et al., 1989, Mayhew et al., 1993, Varastehpour et al., 2006, Vambergue and Fajardy, 2011, Gauster et al., 2012). These outcomes may give rise to, or be a consequence of, impaired fetal oxygenation. Placental weights also tend to be heavier in maternal obesity and diabetes and correlate with fetal overgrowth (Taricco et al., 2003, Swanson and Bewtra, 2008).

An unfavourable maternal environment may therefore have detrimental effects on the developing placenta, which in turn may have adverse effects on the fetus. The placenta can be viewed as a compromised target that largely suffers the impact of a perturbed maternal environment. Diabetes and obesity can alter placental anatomy and physiology, thereby affecting maternal-fetal nutrient transfer, fetal growth, perinatal outcomes and potentially even long-term health of the offspring.

1.5 Studying diabetes in pregnancy in animal models

A range of strategies are available to induce diabetes, hyperglycaemia and insulin resistance in animal models, although there is currently no widely accepted animal model of Type 2 diabetes in pregnancy or of gestational diabetes. The most common and thoroughly described models are in rodents that develop diabetes by spontaneous, chemical, or dietary means, as well as genetic models.

1.5.1 Animal models of Type 1 diabetes in pregnancy

By far the most commonly used method to induce diabetes in pregnancy in rodents involves the administration of Streptozotocin (STZ). STZ is a diabetogenic drug that selectively destroys pancreatic β cells leading to insulin deficiency and hyperglycaemia (Elsner et al., 2000). STZ-induced Type 1

diabetes has been used extensively to study pregnancy complications and fetal outcomes in rats and mice using a range of doses and administration modes at different gestational ages (reviewed by Jawerbaum and White (2010)). Pups of severe STZ-induced diabetes are however typically growth retarded (Aerts et al., 1990, Padmanabhan and Shafiullah, 2001, Lopez-Soldado and Herrera, 2003, Kiss et al., 2009). Mild diabetes in pregnancy has been reported using multiple low-dose STZ protocols but with variable outcomes regarding fetal weight and hyperinsulinaemia and may have limited clinical relevance (Caluwaerts et al., 2003).

Less common are genetic models of Type 1 diabetes which include the non-obese diabetic (NOD) mouse and bio-breeding (BB) rat. These spontaneous models arise from selective inbreeding for hyperglycaemia and share similar immunological origins to human Type 1 diabetes (Rees and Alcolado, 2005). The drawbacks of using these strains in studies of diabetic pregnancy are that NOD mice do not develop insulin-dependent diabetes until 30 weeks of age, and BB rats develop severe diabetes and severe ketoacidosis (Nakhooda et al., 1977, Formby et al., 1987). An additional genetic model of insulin-dependent diabetes is the Akita mouse. Akita mice have an autosomal dominant point mutation in the Insulin II gene and display hyperglycaemia and hypoinsulineamia from 3-4 weeks of age in the absence of obesity (Wang et al., 1999). Surprisingly few studies have utilised Akita mice to model maternal diabetes (Lindegaard and Nielsen, 2008, Grasemann et al., 2012).

1.5.2 Animal models of Type 2 diabetes in pregnancy

Selective inbreeding of certain rodent strains for features of insulin resistance has given rise to the Cohen diabetic rat and the Goto Kakizaki rat. These non-obese polygenic models have been used to assess congenital abnormalities and the programming of disease in the setting of diabetic pregnancy (Zusman and Ornoy, 1986, Gauguier et al., 1994, Malaisse-Lagae et al., 1997, Gill-Randall et al., 2004). Most obese monogenic models of Type 2 diabetes (such as leptin deficient (*db*/*db*) mice, leptin-receptor deficient (*ob*/*ob*) mice and Zucker (*fa*/*fa*) rats) are infertile.

Type 2 diabetes can be induced in rodents by high fat feeding. This method was initially developed by feeding C57BL6 mice a diet high in fat content leading to obesity, hyperglycaemia, hyperinsulinaemia and insulin resistance (Surwit et al., 1988). Maternal Type 2 diabetes can also be modelled by high fat feeding during pregnancy. Animals subject to a high fat diet exhibit increased body weight, caloric intake, and elevated plasma glucose, insulin and leptin levels prior to and throughout pregnancy (Taylor et al., 2003, Samuelsson et al., 2008, Kirk et al., 2009, Nivoit et al., 2009) with offspring showing neonatal macrosomia (Samuelsson et al., 2008).

1.5.3 Animal models of gestational diabetes

A range of models have been reported to mimic the onset of hyperglycaemia and insulin resistance during pregnancy. Offspring of dams with STZ-induced diabetes, uteroplacental insufficiency or protein restriction have altered glucose and insulin profiles in pregnancy and as such are labelled with gestational diabetes (Aerts et al., 1990, Oh et al., 1991, Boloker et al., 2002, Zambrano et al., 2005). Whether these second generation offspring exhibit this phenotype prior to pregnancy or whether the insulin resistant nature of pregnancy brings out this phenotype has not been clearly delineated.

Holemans et al. (2004) reported a novel diet-induced model of gestational diabetes. Wistar rats were found to display normal glucose tolerance prior to pregnancy following 4 weeks on a high calorie cafeteria-style diet, yet in late gestation developed impaired glucose tolerance and a marked elevation in insulin resistance. Mild gestational diabetes has also been modelled using continuous glucose infusion in the pregnant rat in late gestation (Nicolaidis et al., 1974, Ktorza et al., 1981, Gauguier et al., 1991). This involves rodents being fitted with an intracardiac catheter or indwelling jugular vein catheter during gestation. Unrestrained dams are then continuously infused with glucose via a peristaltic pump to maintain mild hyperglycaemia.

An additional model of gestational diabetes with a phenotype that may best resemble the human condition is the leptin receptor deficient, $Lepr^{db/+}$, mouse. Leptin is a hormone produced primarily by adipocytes that provides negative feedback between adipose tissue and the hypothalamic satiety centre. Leptin signalling regulates energy balance by decreasing food intake and increasing energy expenditure (Munzberg and Morrison, 2015). While homozygous $Lepr^{db/db}$ mice display severe hyperphagia, glucose intolerance and insulin resistance and are infertile, mice carrying a heterozygous mutation in the leptin receptor are reported to spontaneously develop glucose intolerance in late gestation. $Lepr^{db/+}$ mice exhibit hyperphagia, increased weight gain and elevated glucose and insulin levels in late gestation compared to wild type mice, differences that are not observed prior to pregnancy (Kaufmann et al., 1981, Ishizuka et al., 1999, Yamashita et al., 2003, Stanley et al., 2011). Moreover, $Lepr^{db/+}$ mice have an increased prevalence of macrosomic offspring (Kaufmann et al., 1981, Lawrence et al., 1989, Yamashita et al., 2001, Yamashita et al., 2003, Lambin et al., 2007, Stanley et al., 2011).

1.5.4 Developmental programming in animal models of diabetic pregnancy

It is well recognised that offspring of diabetic pregnancy have an increased risk of obesity, glucose intolerance, diabetes and hypertension in adulthood. These outcomes have also been reported in animal studies. Adult male offspring of *db*/+ mice exhibit greater weight gain (Lambin et al., 2007) while female offspring have greater percentage body fat and hyperinsulinaemia (Yamashita et al.,

2003) than offspring of wild type dams. It is unclear why these sex differences are present. Offspring of STZ-induced diabetic pregnancy subject to mild hyperglycaemia show deficient insulin secretion whereas offspring of severe hyperglycaemia exhibit elevated insulin secretion and insulin resistance (Aerts et al., 1990). Offspring of STZ-diabetic pregnancy may also be overweight or small for their age in adulthood, respective to mild or severe maternal hyperglycaemia exposure (Aerts et al., 1990, Rocha et al., 2005, Nehiri et al., 2008, Chen et al., 2010).

The most consistent data pertaining to developmental programming following a diabetic pregnancy comes from studies of animals fed a high fat or high fat/high sucrose diet to induce diabetes in pregnancy. Adult offspring of these dams exhibit greater weight gain, higher percentage body fat, impaired glucose tolerance, hypertension and altered insulin responses; features indicative of metabolic syndrome and consistent with that seen in offspring of diabetic pregnancy in the clinical situation (Khan et al., 2003, Srinivasan et al., 2006a, Samuelsson et al., 2008, Elahi et al., 2009, Järvelin et al., 2009, Liang et al., 2009b, Nivoit et al., 2009, Oben et al., 2010, Samuelsson et al., 2013, Masuyama and Hiramatsu, 2014). The offspring phenotype reported in these studies may be a direct effect of a maternal high energy diet or may be a consequence of maternal obesity and increased fat mass *per se*. The relative contribution of maternal hyperglycaemia and hyperinsulinaemia are also unclear. It is therefore difficult to delineate the relative contributions of the various factors to offspring disease in models of maternal overnutrition.

1.6 Diabetes in pregnancy and kidney development

As described above, it is well known that diabetes in pregnancy can affect the development of a range of organs and is associated with an increased risk of congenital abnormalities. Of particular relevance to this thesis is the high incidence of urinary tract malformations and renal anomalies. Infants of women with pre-gestational diabetes have a 3-7 fold increased risk of any renal or urinary tract defect (Martinez-Frias, 1994, Sheffield et al., 2002, Shnorhavorian et al., 2011), with studies reporting a 3-15 fold increased risk of renal agenesis, dysgenesis or hypoplasia (Nielsen et al., 2005, Correa et al., 2008, Bánhidy et al., 2010, Davis et al., 2010, Hsu et al., 2014) and a 1.5-5 fold increased risk of obstructive anomalies of the urinary tract (Nielsen et al., 2005, Bánhidy et al., 2010, Shnorhavorian et al., 2011). Cohort studies have also identified abnormalities of the renal/urinary system in infants of gestational diabetic women, although it is important to acknowledge these studies may include women with unrecognised pre-existing Type 2 diabetes (Aberg et al., 2001). Two studies report a 40-80% increase in any renal/urinary anomaly in infants of gestational diabetes (Martínez-Frías et al., 1998, Shnorhavorian et al., 2011). Studies that assess specific kidney and urinary tract malformations report no increased risk of renal agenesis, dysplasia or hypoplasia (Correa et al., 2008, Bánhidy et al., 2010,

Hsu et al., 2014), but a 1.5-5 fold increased risk of obstructive urinary tract congenital malformations (Kamdem et al., 2005, Bánhidy et al., 2010, Hsu et al., 2014). Gestational diabetes is also a risk factor for ureteropelvic junction syndrome and vesicoureteric reflux (Kamdem et al., 2005). The underlying cause of renal and urinary tract defects in diabetic pregnancy is unclear, but is hypothesised to be mediated by the teratogenic effects of hyperglycaemia, ketones and ROS which in turn may alter key developmental pathways in the kidney and potentially alter the fetal epigenome (Renkema et al., 2015).

Recently, maternal obesity has also been associated with renal birth defects independent of maternal diabetes. Hsu et al. (2014) identified a 25% greater risk of obstructive uropathy and chronic kidney disease (CKD) in children of overweight and obese women. Similarly, Macumber and Leca (2014) reported a positive association between maternal obesity and risk of infant renal anomaly. These findings were not attenuated after adjusting for maternal diabetes, and demonstrate that maternal obesity may also independently perturb development of the kidney and urinary tract.

1.7 An overview of kidney development

As the kidney is susceptible to altered development in maternal diabetes, it is imperative to determine whether hyperglycaemia in pregnancy influences key processes of kidney development. A timeline of kidney development in the mouse and human is outlined in **Table 1.4**.

Table 1.4 Timing of kidney development and nephrogenesis in the human and mouse

	Human	Mouse
Pronephros		
First appears	22 days	8 days
Regresses by	25 days	9 days
Mesonephros		
First appears	24 days	10 days
Regresses by	16 weeks	14 days
Metanephros appears	28-32 days	10.5 days
Renal pelvis	33 days	12 days
Collecting tubules	44 days	13 days
Nephrogenesis begins	9 weeks	12 days
Nephrogenesis ceases	34-36 weeks	Postnatal day 5
Length of gestation	40 weeks	20 days

(Moritz and Wintour, 1999, Shah et al., 2004)

1.7.1 The pronephros, mesonephros and metanephros

Mammalian kidney development involves the sequential formation of three pairs of renal organs derived from the intermediate mesoderm: the pronephros, mesonephros and metanephros. The

pronephros and mesonephros are transient precursors of the metanephros, the adult kidney. The metanephros is a permanent and fully functional excretory organ which develops caudal to the mesonephros. Metanephric development is initiated by the interaction of two tissue types: the epithelial ureteric bud that projects from the caudal nephric duct, and the surrounding metanephric mesenchyme (Clark and Bertram, 1999). The ureteric epithelial bud branches to form the renal collecting duct system as well as the renal calyces, renal pelvis and ureter, while the metanephric mesenchyme gives rise to nephrons. These processes are regulated by a series of inductive signals and reciprocal interactions between the two precursor cell populations.

1.7.2 The ureteric bud and branching morphogenesis

Ureteric budding and branching morphogenesis are highly complex yet elegant processes with four general developmental stages: (i) outgrowth of the ureteric bud from the caudal nephric duct; (ii) rapid, iterative branching of the ureteric tree; (iii) deceleration of ureteric branching morphogenesis and differentiation of metanephric mesenchyme; and (iv) termination of branching and completion of mesenchymal differentiation and nephron formation (Cebrián et al., 2004, Nigam and Shah, 2009).

Ureteric bud outgrowth and early branching morphogenesis are illustrated in **Figure 1.1**. The initial outgrowth of the ureteric bud is mediated principally by Ret/Glial cell-line derived neurotrophic factor (GDNF) signalling. GDNF is expressed by the undifferentiated metanephric mesenchyme and signals through the receptor Ret (a tyrosine kinase receptor) and the co-receptor GFRα1 (the GDNF family receptor α1), both of which are expressed along the Wolffian duct (Michos, 2009). Activation of the Ret receptor adjacent to the metanephric mesenchyme stimulates epithelial cell migration and proliferation, and evagination of the ureteric bud (Dressler, 2006). The positioning of the ureteric bud outgrowth is also regulated by other genes and signalling systems, including bone morphogenetic protein 4 (BMP4), gremlin, Slit2/Robo2 and Sprouty1 (Michos, 2009). Full or partial knockdown of these genes can result in ectopic budding of the ureteric bud.

Once the ureteric bud projects from the Wolffian duct and invades the metanephric mesenchyme it begins to branch, a process termed ureteric branching morphogenesis. At this stage, Ret-GFRα1 expression is restricted to the distal tips of the ureteric bud while GDNF expression is limited to the mesenchyme surrounding each ureteric bud tip (Costantini and Shakya, 2006). This defines the trunk of the bud (Ret negative) from the highly proliferating ureteric tips (Ret positive). Upon instruction from the surrounding mesenchyme which stimulates ureteric epithelial cell proliferation, each ureteric bud tip undergoes bifurcation into two or sometimes three segments (Moritz and Wintour, 1999). These new branches elongate and branch again at the tips in a repetitive process. In humans, ureteric branching is thought to occur approximately 15 times and in mice approximately 12 times (al-Awqati

and Goldberg, 1998, Cebrián et al., 2004, Short et al., 2014). The accelerative stage of ureteric branching morphogenesis is driven by positive feedback signalling. Correct positioning of Ret and the progression of branching morphogenesis is assured by stimulatory molecules (heparan sulphate proteoglycans, Wnt11, fibroblast growth factors (FGF), epidermal growth factor (EGF) and hepatocyte growth factor (HGF)) and inhibitory molecules (Sprouty1, transforming growth factor-β1 (TGF-β1), BMP4 and activin) that demonstrate a temporal-spatial expression pattern (Michos, 2009). Later deceleration of ureteric branching occurs due to a predominance of negative feedback signalling. The cessation of ureteric branching morphogenesis seems to correlate with the differentiation of metanephric mesenchyme, and signals from the ureteric epithelium that promote nephron formation inhibit further branching. Ultimately, the dichotomous branching of the ureteric epithelium forms the collecting duct system. Expansion and remodelling of early branch generations forms the renal calyces, renal pelvis and ureter (Woolf et al., 2003).

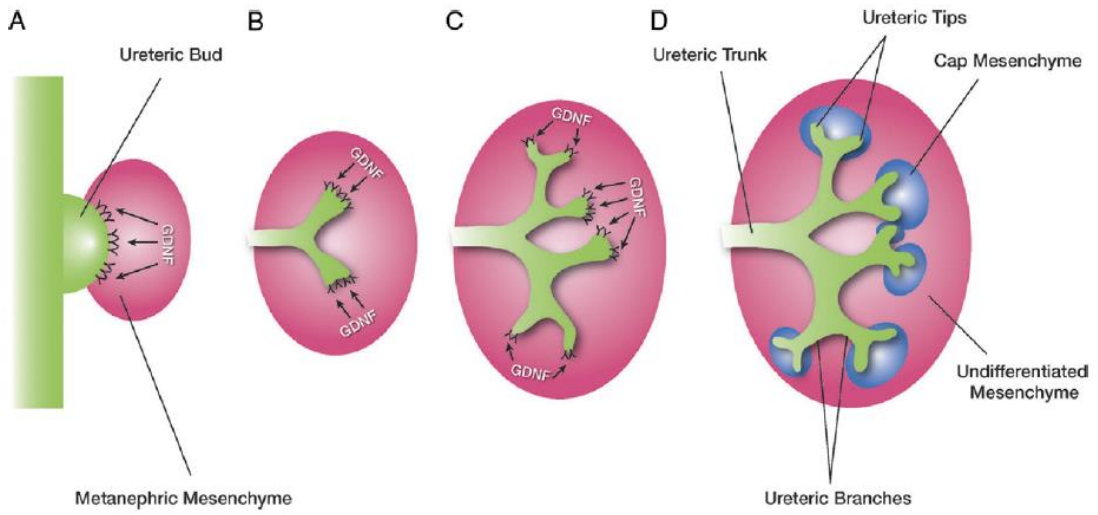


Figure 1.1 Ureteric budding and ureteric branching morphogenesis
A GDNF is secreted by metanephric mesenchyme and acts directly on Ret-GFRα1 receptor complexes expressed by the epithelium (Y) to initiate invasion of the metanephric mesenchyme (pink) by the ureteric bud (green). **B** The ureteric bud then undergoes a dichotomous branching event termed T-stage. **C** Ret-GFRα1 expression becomes localised to ureteric tips, restricting branching events to the tips of developing ureteric branches. **D** Subsequent dichotomous branching events give rise to the ureteric tree, with mesenchymal cells induced to condense and form cap mesenchyme at the ureteric tips. Figure adapted from Walker and Bertram (2011).

Outgrowth and correct positioning of the ureteric bud is essential as defects in this process account for the majority of congenital anomalies of the kidney and urinary tract including renal agenesis, renal dysgenesis and vesicoureteral reflux. Ureteric branching morphogenesis not only establishes the architecture of the kidney but is also a key determinant of nephron number as nephron formation

occurs adjacent to ureteric tips (Nigam and Shah, 2009). Deficiencies in ureteric branching morphogenesis may therefore lead to renal dysplasia, renal hypoplasia or a low nephron endowment.

1.7.3 Nephrogenesis

As new branch tips are generated in the developing metanephros, and as the rate of ureteric branching slows, reciprocal signalling between the ureteric epithelial tips and the surrounding metanephric mesenchyme induce nephron formation. This process, termed nephrogenesis, requires mesenchymal cell condensation and mesenchymal-to-epithelial conversion (Clark and Bertram, 1999). A schematic representation of the stages of nephrogenesis is presented in **Figure 1.2**. Nephrogenesis occurs at the periphery of the kidney in the nephrogenic zone. Here, signalling from each ureteric branch tip induces surrounding mesenchymal cells to condense and form nephron progenitor cells, termed cap mesenchyme (**Figure 1.2B**). Cap mesenchyme cells support, promote and regulate ureteric branching and induce the formation of pretubular aggregates proximal to the ureteric tip (**Figure 1.2C**) (Walker and Bertram, 2011, Cebrian et al., 2014). Pretubular aggregates then undergo mesenchyme-to-epithelial conversion to form a renal vesicle which will give rise to all epithelial cells of the nephron. Next, a cleft forms in the renal vesicle to give rise to a comma-shaped body which further differentiates into an S-shaped body (**Figure 1.2E-F**). The upper portion of the S-shaped body forms the distal convoluted tubule, the middle portion forms the distal straight tubule, loop of Henle and proximal tubule, and the lower portion forms the renal corpuscle. Cells of the inner epithelium of the lower cleft differentiate into glomerular podocytes, while the parietal epithelial cells of Bowman's capsule are formed from the outer epithelial lining (Moritz et al., 2008, Walker and Bertram, 2011). At this stage, the distal end of the nephron has fused to the collecting duct to form one continuous tubule while the proximal end is infiltrated with endothelial cells which form the glomerular capillary network (**Figure 1.2H**). Podocytes differentiate to produce filtration slit diaphragms and foot processes, while fusion of the basement membranes of podocytes and glomerular endothelial cells forms the glomerular basement membrane.

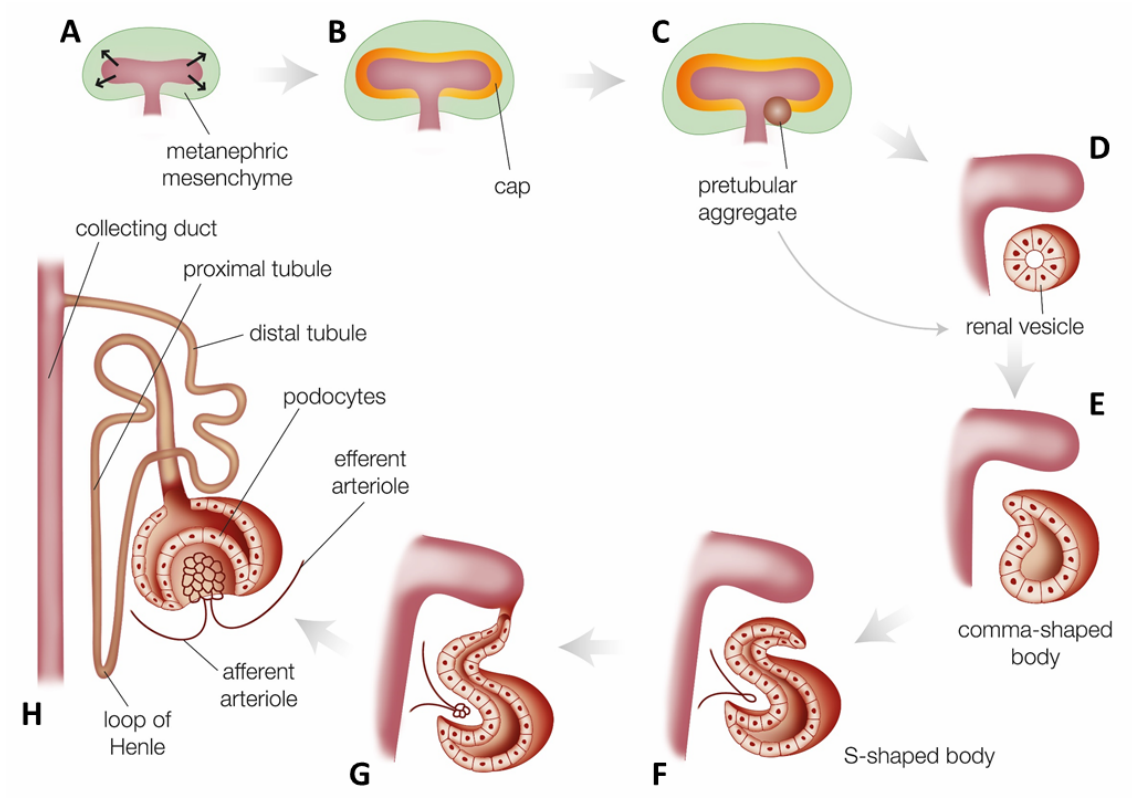


Figure 1.2 The stages of nephrogenesis.
A Signals from the metanephric mesenchyme induce branching of the ureteric bud. **B** Signals from the ureteric branch tips induce adjacent mesenchyme to condense and form cap mesenchyme, which induces the formation of **C** a pretubular aggregate. **D** This pre-tubular aggregate undergoes mesenchyme-epithelial conversion to form a renal vesicle, **E** comma-shaped body and **F** S-shaped body. **G** At this stage, endothelial cells migrate into the distal cleft of the S-shaped body to form the glomerulus and the upper limb fuses to the future collecting duct. **H** The lower limb of the S-shaped body gives rise to Bowman's capsule and podocytes, cells that together with endothelial cells and the glomerular basement membrane form the filtration barrier. Filtrate passes into Bowman's space, through to the proximal tubule, loop of Henle and distal tubule, formed from the upper limb of the S-shaped body. Figure modified from Moritz et al. (2008).

The quantitative relationship between ureteric branching and nephron number is not consistent throughout renal development. During the initial stages of branching morphogenesis, the ureteric epithelium gives rise to the renal pelvis and calyces and few nephrons form (Moritz et al., 2008). From then, each tip induces the formation of one nephron until approximately 15 weeks gestation (**Figure 1.3A**). Studies in mice show that 85% of total branching events have taken place by embryonic day (E) 16.5 (Cebrián et al., 2004). After these time points, ureteric branching slows, the distance between peripheral ureteric branch points increases, and the rate of nephrogenesis increases. These nephrons are arranged in arcades whereby a single elongating tip repeatedly induces new nephrons (**Figure 1.3B-C**). As nephrons are formed along the arcade, the connecting tubule of the older nephron merges from the ureteric tip to the collecting tubule of the newer nephron resulting in several nephrons attached

to the same collecting tubule (Moritz et al., 2008). The process of arcade formation occurs between approximately 15-22 weeks of gestation in humans, with four to seven nephrons formed in each arcade (Osathanondh and Potter, 1963). Studies in rats have found nephron arcades to form between E17 and postnatal day (PN) 3, with three to four nephrons per arcade (Neiss, 1982). Studies in mice are lacking but considering similarities in gestation and kidney development between the mouse and rat, it can be approximated that nephron arcades form between E15 and PN1 in mice. The formation of arcades positions nephrons in the inner cortex of the mature kidney. Nephrons are positioned in the outer cortex by late-phase terminal branching and lateral elongation. Here, ureteric branches elongate into the outer cortex and induce the formation of multiple nephrons, each with a direct attachment to the stem of the collecting duct (**Figure 1.3**D) (Clapp and Croker, 2012). This phase of nephron formation occurs between approximately 22 weeks and 34-36 weeks gestation, with the majority of nephrons formed in the third trimester in humans and in the early postnatal period in mice. In humans, nephrogenesis is complete by week 34-36 of gestational and in mice it is complete by approximately PN5, after which no new nephrons can form (Hinchliffe et al., 1991, Sims-Lucas et al., 2008).

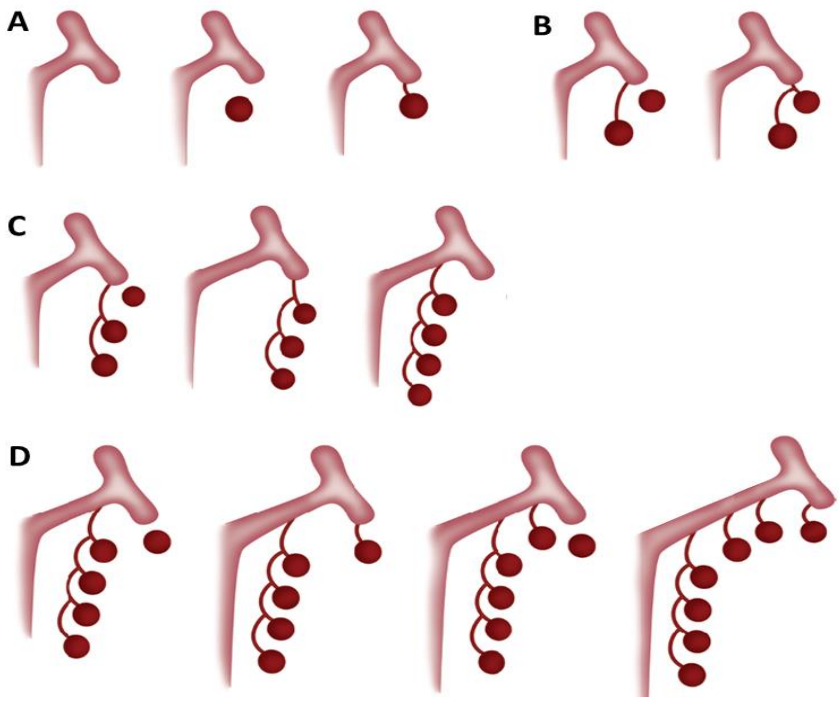


Figure 1.3 The relationship between ureteric branching and nephron formation
Nephron endowment is considered to be largely determined through branching and elongation of the ureteric epithelium and branching iterations (arcades). **A** During early nephrogenesis each ureteric tip induces the formation of one nephron that fuses to the tip (collecting duct). **B** Later in nephrogenesis the same tip can induce the formation of another nephron, which attaches to the ureteric tip and the connecting tubule of the older nephron shifts to the new nephron. **C** This process continues until four to seven nephrons form an arcade. **D** Nephrons also attach along the entire length of the collecting duct as it elongates in the outer cortex. Figure adapted from Moritz et al. (2008) and Osathanondh and Potter (1963).

Autopsy studies have identified a remarkably wide range in nephron number in human kidneys without overt kidney disease. A large study of 398 kidneys of subjects from five racial groups identified an approximate 13-fold range in human nephron number (Bertram et al., 2011). These estimates varied from 210,332 to 2,702,079 nephrons per kidney with a mean of 895,711 nephrons.

1.8 Kidney development and programming of adult disease

Since the early enunciation of the fetal origins hypothesis by Barker, an extensive body of evidence has emerged to support the concept that prenatal environmental factors can increase predisposition to adult disease. Several studies have reported altered renal development in animal models of developmental programming whereby the offspring are subject to suboptimal intrauterine environments such as food restriction, protein restriction, and exposure to corticosteroids and antibiotics (reviewed by Lelièvre-Pégorier and Merlet-Bénichou (2000), Kett and Bertram (2004) and Hoy et al. (2010)). The kidney is thought to be particularly susceptible to an adverse intrauterine

environment because, in humans, development occurs over a long period of gestation and nephrogenesis is complete before birth. Once nephrogenesis ceases, any deficit in nephron endowment is permanent.

The significance of a nephron deficit on adult health was proposed by Brenner et al. (1988), who hypothesised an association between low nephron number and the development of renal disease and hypertension. According to the Brenner hypothesis, low nephron endowment (either congenital or acquired) leads to glomerular hyperfiltration and hypertension to maintain renal blood flow, with the consequence of glomerular injury (including glomerulosclerosis), proteinuria and systemic hypertension. Glomerulosclerosis can also lead to further nephron loss, resulting in a cyclical process (**Figure 1.4**).

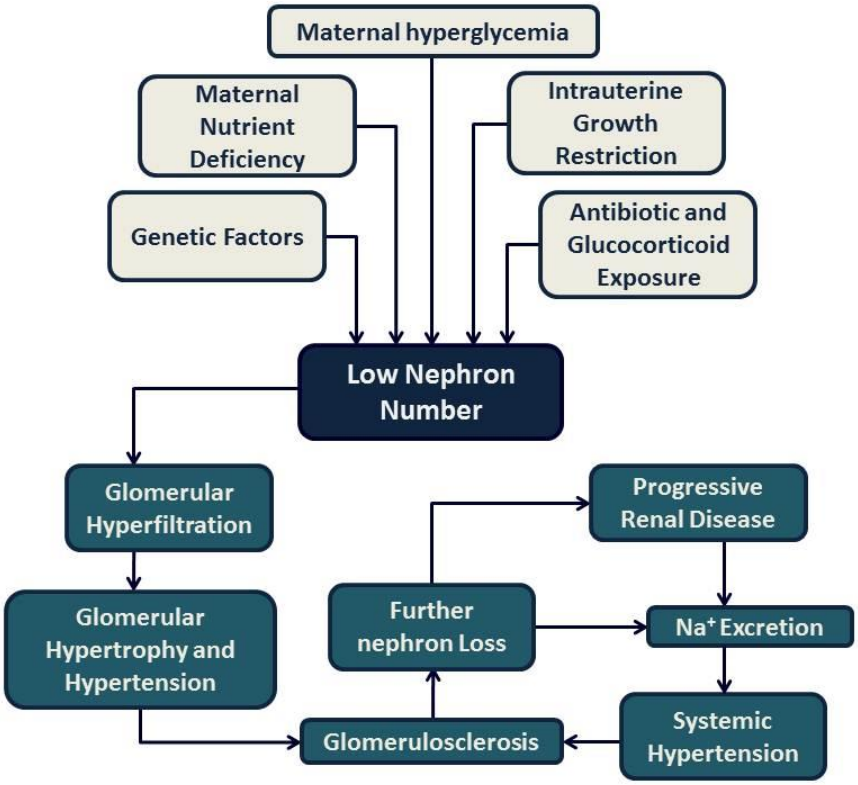


Figure 1.4 Schematic diagram of the 'Barker Developmental Programming Hypothesis' and 'Brenner Hypothesis'

Additive to the genetic background, the developing fetus can be exposed to numerous intrauterine factors that programme a permanent reduction in nephron number. Low nephron endowment can lead to the development of adult disease. Glomerular hyperfiltration and hypertrophy occur to maintain renal blood flow, with the consequence of glomerular hypertension and glomerulosclerosis. In a cyclical process this can lead to further nephron loss, systemic hypertension and further renal injury.

This hypothesis is supported by numerous experimental studies that have identified a range of maternal/intrauterine factors and genetic mutations associated with a congenital nephron deficit and increased blood pressure in offspring (reviewed by Kett and Bertram (2004), Moritz et al. (2009) and Chong and Yosypiv (2012)). Emerging evidence also indicates that other factors, in addition to a nephron deficit, may also contribute to developmental programming of renal dysfunction and hypertension in an adverse intrauterine environment. These include alterations in renal tubule sodium transporters, vascular function, and activity of the renin-angiotensin-aldosterone system and sympathetic nervous system (Benz and Amann, 2010, Luyckx and Brenner, 2010). While there is some contention regarding the Brenner hypothesis due to the absence of hypertension in unilateral nephrectomised animals and human kidney donors (Kett and Bertram, 2004, Ibrahim et al., 2009), several studies in Caucasian populations have reported an inverse relationship between the incidence of hypertension and nephron number (Keller et al., 2003, Hughson et al., 2006, Puelles et al., 2011). Hypertension is one of the most common diseases in the Western world (Lim et al., 2012), and in addition to genetic and lifestyle factors a low nephron endowment at birth due to programming events may be a risk factor for hypertension and renal disease in adulthood. It is therefore important to gain an understanding of how intrauterine events such as hyperglycaemia may alter kidney development and in turn enhance susceptibility to adult disease.

1.9 Kidney development in offspring of diabetic pregnancy

While human diabetic pregnancy is associated with an increased risk of renal and urinary tract malformations, less severe outcomes such as altered nephron endowment following exposure to intrauterine hyperglycaemia have not been described. The lack of human data in this field is not surprising given that currently, human nephron number can only be measured from autopsy tissue. Recently, imaging techniques have been applied to assess kidney volume, as a proxy of renal sufficiency, in offspring of diabetic women. A small study by Cappuccini et al. (2013) found that 3 year old children of Type 2 diabetic and gestational diabetic women had reduced renal cortical volume. Conversely, a longitudinal study by Neves et al. (2013) found increased fetal renal volume in gestational diabetes. To date, much of our understanding of the effect of hyperglycaemia on kidney development stems from animal studies.

1.9.1 *In vitro* studies

Initial studies investigating kidney development in a high glucose environment were conducted using embryonic kidney explants cultured for 6-7 days in high glucose media. Culture studies by Kanwar et al. (1996) and Amri et al. (1999) reported branching dysmorphogenesis, blunt ureteric tips and poor arborisation of the ureteric tree in a high glucose environment. These observations were coupled with

reduced nephrogenesis highlighting the dependence of nephron formation on the branching capacity of ureteric epithelia. More recently, Zhang et al. (2007) observed a stimulatory effect of comparably high but transient glucose on ureteric branching in whole cultured mouse metanephroi. These discrepancies in findings are likely the result of variability inherent to culture preparations and differences in the duration of exposure, glucose concentration, length of culture and the type of media and supplements used.

1.9.2 *In vivo* studies

In vivo studies have further elucidated the effects of hyperglycaemia on ureteric branching morphogenesis and nephrogenesis. These studies have primarily utilised STZ-induced diabetes in rodents. Studies in our laboratory and others show that embryos of STZ-treated dams have a 30-50% reduction in ureteric tree development, specifically ureteric tip number, tree length and tree volume with greater deficits in branching morphogenesis associated with longer exposure to maternal hyperglycaemia (Chang et al., 2012, Hokke et al., 2013). Congenital renal abnormalities including duplex ureter and duplex collecting duct systems have also been observed in offspring of diabetic dams, suggesting ureteric budding and early ureteric branching morphogenesis are primary processes targeted in hyperglycaemia (Hokke et al., 2013).

Nephrogenesis is also perturbed in maternal hyperglycaemia, with studies reporting a 15-40% deficit in nephron number in offspring of STZ-induced diabetic dams (Amri et al., 2001, Tran et al., 2008, Dezfoolian et al., 2009, Chen et al., 2011, Hokke et al., 2013). Together, these publications demonstrate that diabetic pregnancy impairs nephrogenesis in proportion to the magnitude of maternal glycaemia. Interestingly, studies in our own laboratory (Hokke et al., 2013) found maternal insulin treatment administered in late gestation could not prevent a deficit in nephron number, despite restoring maternal glucose levels. This highlights how a window of hyperglycaemia during early kidney development can lead to a permanent deficit in nephron formation, and further implicates ureteric branching as a primary process affected by hyperglycaemia. Chen et al. (2011) also administered insulin in late gestation and reported partial normalisation of maternal glucose levels and nephron number in neonates of STZ-induced dams. In this study, insulin was initiated 2 days after STZ treatment, whereas in Hokke et al. (2013) insulin was given 7 days after STZ administration. The discrepancy between these two studies is likely related to the timing of diabetes induction and insulin initiation, with a longer window of glucose exposure before insulin treatment associated with a greater deficit in offspring nephron number.

1.9.3 Limitations of previous studies

Reduced nephron endowment has been reported in normal birth weight offspring of STZ-induced hyperglycaemia in pregnancy (Amri et al., 1999). However, the majority of animal studies report a nephron deficit in conjunction with growth restriction (Amri et al., 1999, Tran et al., 2008, Dezfoolian et al., 2009, Hokke et al., 2013). In these studies, offspring growth restriction correlated with the degree of maternal hyperglycaemia: dams administered a mild dose of STZ displayed blood glucose levels of 18mmol/l and normal pup weight, whereas dams administered a higher dose of STZ displayed blood glucose levels up to 25mmol/l and offspring growth restriction. Growth restriction is not a frequent neonatal outcome of human diabetic pregnancy and is typically seen in only a small subset of pregnancies complicated by pre-eclampsia or vascular disease (Nold and Georgieff, 2004). A significant positive linear relationship has been identified between embryo weight and kidney development in both control and growth restricted offspring of diabetic pregnancy (Amri et al., 1999, Tran et al., 2008, Hokke et al., 2013). Kidney development in LGA offspring as seen in the clinical situation is currently unknown. Whether kidney development in these offspring follows a positive linear relationship, or whether LGA offspring have an absolute or relative deficit in kidney development with respect to body weight has yet to be investigated.

Published studies assessing kidney development in diabetic pregnancy have, with the exception of one glucose infusion model (Amri et al., 1999), been based on the STZ-induced Type 1 diabetic model of severe hyperglycaemia. To date, no animal study has investigated the impact of impaired glucose tolerance or insulin resistance in pregnancy, be it pre-gestational Type 2 diabetes or gestational diabetes, on fetal renal development. Analysis of kidney development in animal models that better mimic the human situation of mild-moderate hyperglycaemia, insulin resistance, postprandial hyperglycaemic excursions and associated fetal macrosomia are required.

1.10 Mechanisms of altered kidney development in hyperglycaemia

The majority of studies analysing how kidney development is affected in hyperglycaemia have focused on the impact on nephron endowment as an insight into the efficiency of renal development. Final nephron number depends on a multitude of processes including ureteric budding, ureteric branching morphogenesis, mesenchymal-epithelial conversion, apoptosis and maintenance of nephron structures (Abrahamson and Steenhard, 2008). Just how a high glucose environment can interfere with these processes is poorly understood. Research to date has focused on ROS and their role in apoptosis and cellular interactions, as well as IGFs.

1.10.1 Reactive oxygen species

In a high glucose environment ROS generation is increased due to oxidative phosphorylation and superoxide production (Kanwar et al., 2005). In the fetal environment this can be heightened by the relative immaturity of the free radical scavenging system. ROS have been implicated as teratogens in diabetic embryopathy leading to congenital malformations, as discussed in **Section 1.2.1**. Several studies have also associated ROS with altered renal development. *In vitro*, MK4 cell lines (representative of late metanephric mesenchyme) and whole embryonic kidney explants cultured in high glucose exhibit heightened superoxide generation (Chen et al., 2006, Zhang et al., 2007). Elevated superoxide production has also been demonstrated *ex vivo* in neonatal kidneys of STZ-induced diabetes and, moreover, has been associated with a deficit in nephron endowment (Tran et al., 2008, Chen et al., 2011). The direct effect of renal ROS induced in maternal diabetes was further investigated by Chang et al. (2012) who overexpressed catalase, a key antioxidant enzyme regulating hydrogen peroxide levels, in renal proximal tubule cells of neonatal kidneys. Catalase overexpression in the presence of STZ-induced maternal diabetes attenuated deficits in ureteric branching morphogenesis and nephrogenesis.

Oxygen free radicals can directly damage DNA, modify and induce structural damage in extracellular matrix (ECM) macromolecules and alter the expression profile of extracellular glycoproteins to induce damage in several organ systems. The mechanisms whereby elevated ROS can induce alterations in renal development are however poorly understood (Kanwar et al., 2005). As described in **Section 1.7.3**, during nephrogenesis epithelial/mesenchyme interactions are required at the interface of ureteric tips and nephron progenitor populations to maintain cell-cell contacts and reciprocal signalling interactions. Embryonic kidneys cultured in high glucose are reported to have reduced gene and protein expression of heparan sulphate proteoglycan, a component of the ECM, together with branching morphogenesis and atrophy of mesenchyme populations (Kanwar et al., 1996). This highlights how elevated glucose may disrupt epithelial/mesenchymal cell interactions to affect branching of the ureteric tree and, in turn, nephrogenesis.

1.10.2 Apoptosis

Elevated apoptosis has been identified in kidneys exposed to a hyperglycaemic environment. Embryonic kidneys cultured in high glucose with elevated ROS production not only display atrophic mesenchyme but also show elevated apoptosis and reduced proliferation of mesenchymal cell populations (Kanwar et al., 1996). *In vivo*, kidneys of neonatal pups exposed to maternal STZ-induced diabetes have increased apoptotic markers in podocytes and renal tubular cells and are associated with nephron collapse and a reduction in nephron number (Tran et al., 2008, Chen et al., 2011).

Increased apoptosis, seen in the ureteric epithelium and nascent glomeruli, is reported to be mediated by oxidative stress via activation of NFκB signalling, a recognised pathway of inflammatory responses and apoptosis (Chen et al., 2006, Chen et al., 2011).

1.10.3 Insulin growth factors

Insulin, IGFs and their receptors are crucial for normal metanephric development. *In vitro* studies show that insulin has a hypertrophic effect on kidney growth and nascent nephrons (Liu et al., 1997). Furthermore, inhibition of IGF-I, IGF-II, insulin receptor (IR) and IGF-I receptor (IGF-IR) is associated with reduced kidney growth and nephron formation as well as disorganised ureteric branching (Liu et al., 1993, Doublier et al., 2001, Duong Van Huyen et al., 2003). This, in turn, is linked with decreased *de novo* synthesis of ECM proteins, implicating the IGF system in mediating epithelial-mesenchyme interactions during early metanephric development.

The IGF system has been studied in the developing fetal kidney exposed to maternal diabetes. Kidney explants from offspring of STZ-diabetic dams show an increase in IGF-II receptor (IGF-IIR) production in ureteric epithelium, committed metanephric mesenchyme and S-shaped bodies (Amri et al., 2001). As this receptor is essential for regulating IGF-II levels, the authors concluded that increased expression of IGF-IIR may lead to reduced bioavailability of IGF-II, a ligand known to stimulate metanephric growth and nephron formation (Gilbert et al., 1998). How a hyperglycaemic intrauterine environment may alter the fetal renal IGF system has not been reported.

1.11 Renal function in offspring of diabetic pregnancy

CKD is characterised by a progressive loss in renal function. One in nine Australians show at least one clinical sign of CKD (AIHW, 2009). Moreover, 35% of new cases of kidney disease have diabetes and an additional 14% of new cases have hypertensive vascular disease. While diabetes is clearly associated with renal disease, termed diabetic nephropathy, the impact of intrauterine hyperglycaemia on adult renal function is poorly understood.

Pregnant women are typically not screened for gestational diabetes until 24-28 weeks gestation (ADA, 2014, ADIPS, 2014). As kidney development begins at 5 weeks gestation with the full complement of nephrons reached by approximately 34-36 weeks, the developing kidney may be exposed to a lengthy period of hyperglycaemia. Impaired kidney development may therefore already be established at diagnosis and treatment interventions may have limited potential to mediate fetal kidney development, as observed in insulin-treated STZ models of diabetic pregnancy (Chen et al., 2011, Hokke et al., 2013). Impaired kidney development (altered ureteric branching morphogenesis, nephrogenesis, or both) may lead to a permanent nephron deficit. This may predispose offspring to hypertension, reduced renal function and renal disease, as first proposed by Brenner et al. (1988).

Exposure to intrauterine hyperglycaemia predisposes the individual to metabolic disorders such as diabetes and obesity, as previously discussed, that may further damage the kidney. Obesity and diabetes are recognised risk factors for CKD, with greater BMI, glucose intolerance and insulin resistance positively and independently associated with glomerular hypertrophy, hyperfiltration and glomerulosclerosis (Eknoyan, 2007, Griffin et al., 2008, Tobar et al., 2013). An altered metabolism may therefore place increased functional demand on the kidney without the supporting renal structure and the long-lasting effects of low nephron endowment may become more clinically apparent.

While no human study has evaluated kidney development in maternal hyperglycaemia, a small number of studies assessing offspring renal function in adulthood have been reported. These studies indicate an increased risk of renal disease and reduced renal function in offspring previously exposed to a diabetic intrauterine environment.

1.11.1 Human studies of renal function

Studies in Pima Indians have reported increased risk of elevated urinary albumin excretion and end stage renal disease (ESRD) in offspring of diabetic mothers. Nelson et al. (1998b) reported a 4-fold increased prevalence of elevated albuminuria in diabetic offspring of diabetic mothers compared to diabetic offspring of non-diabetic and pre-diabetic mothers (later developed diabetes after the pregnancy). Furthermore, a higher prevalence of elevated urinary albumin excretion has been reported in high birth weight Pima offspring, explained largely by the presence of maternal diabetes during pregnancy (Nelson et al., 1998a). Pima Indians with Type 2 diabetes exposed to diabetic pregnancy have a 4-fold increased incidence of ESRD than non-exposed diabetic offspring (Pavkov et al., 2010). This was associated with an earlier diagnosis of diabetes in exposed individuals. Although nephron endowment in these offspring is unknown, it may be that reduced renal development coupled with metabolic dysfunction exacerbates the progression of altered renal function and renal disease.

Renal function has also been assessed in non-diabetic offspring exposed to intrauterine hyperglycaemia. In a small French study, offspring of Type 1 diabetic mothers were compared to offspring of Type 1 diabetic fathers, with offspring of diabetic mothers exhibiting reduced renal functional reserve (Khalil et al., 2010). No difference was found for urinary albumin excretion, but under stimulated conditions offspring had reduced glomerular filtration rate (GFR) and effective renal plasma flow (eRPF) which may reflect a reduced nephron endowment. As mentioned previously, a small study by Cappuccini et al. (2013) found microalbuminuria in 3 year old children of women with Type 2 diabetes and gestational diabetes. This was reported in conjunction with reduced renal cortical volume which may indicate a reduced nephron endowment. The authors suggested that increased

urinary albumin excretion may be related to increased single nephron GFR due to a nephron deficit, however further studies in a larger cohort are required to confirm this finding.

1.11.2 Animal studies of renal function

Rodent studies investigating renal function in offspring exposed to intrauterine diabetes support findings in humans. Offspring of STZ-induced pregnancy exhibit proteinuria and reduced GFR and eRPF (Rocha et al., 2005, Nehiri et al., 2008, Chen et al., 2010). Furthermore, offspring display altered sodium excretion and elevated systolic blood pressure. Glomerular hypertrophy and signs of glomerular sclerosis and interstitial fibrosis have also been reported.

Chang et al. (2012) reported hypertension, proteinuria, glomerular hypertrophy and ECM accumulation in adult offspring with a congenital nephron deficit exposed to maternal diabetes. The authors also found that overexpression of catalase (a key antioxidant enzyme responsible for the degradation of hydrogen peroxide) in renal proximal tubule cells during nephrogenesis attenuated the phenotype in adult offspring. Catalase overexpression was also associated with normalisation of nephron number in these offspring. This highlights that the perinatal programming induced by maternal diabetes was a consequence of low nephron number. Interestingly, other studies of maternal diabetes describe programming of renal disease in rodents both with (Nehiri et al., 2008, Chen et al., 2011) and without (Rocha et al., 2005) a congenital nephron deficit, although Rocha et al. (2005) did report a nephron deficit in aged animals indicating accelerated nephron loss. Other mechanisms, besides altered nephrogenesis, may therefore contribute to long-term consequences of maternal diabetes on renal function and structure. Possible mechanisms include altered vascular reactivity as well as perturbations in renal Na^+,K^+-ATPase and the renin-angiotensin system (Armitage et al., 2005, Rocha et al., 2005).

Collectively, the studies described above provide evidence for fetal programming of the kidney in offspring exposed to *in utero* hyperglycaemia (Rocha et al., 2005, Nehiri et al., 2008, Chen et al., 2010, Chang et al., 2012). The literature is however restricted to models of severe hyperglycaemia induced in the mother by STZ. Renal function in animals exposed to glucose intolerance, mild hyperglycaemia and born LGA, as observed clinically, has yet to be reported. Assessing the relationship between the degree of maternal hyperglycaemia and offspring renal development and adult renal function will provide additional insights into the effect of diabetes in pregnancy.

1.12 Summary

Maternal glucose intolerance is an increasingly common complication of pregnancy that is associated with adverse perinatal outcomes and long-term health implications for offspring. Infants exposed to diabetic pregnancy have an increased risk of congenital malformations of a range of organ systems.

Malformations of the kidneys and urinary tract are particularly prevalent. Few studies, however, have examined the less overt effects of hyperglycaemia on offspring renal development.

Previous experimental studies investigating kidney development and function in maternal diabetes are primarily limited to the paradigm of STZ-induced diabetes. These studies report a deficit in offspring nephron endowment, yet are associated with severe and persistent fasting hyperglycaemia in the dam and offspring growth restriction. STZ studies model the condition seen in only a small subgroup of women with poorly controlled Type 1 diabetes associated with advanced vascular disease and placental complications. For this reason, the effect of maternal diabetes on offspring kidney development, structure and function reported in previous studies has limited applicability, and cannot be directly translated to other more prevalent types of diabetes in pregnancy characterised by maternal glucose intolerance, maternal insulin resistance and fetal overgrowth. To date, no study has assessed fetal kidney development and adult renal function in offspring exposed to maternal glucose intolerance.

Currently, estimation of nephron endowment in humans is only feasible from autopsy tissue. Kidney growth and volume can be measured by ultrasound, however studies assessing kidney development in human pregnancies complicated by maternal diabetes are sparse. These studies reveal both reduced renal cortical volume and increased kidney volume in offspring of women with Type 2 diabetes and/or gestational diabetes. Further studies investigating fetal kidney volume in diabetic pregnancy are evidently required.

1.13 Hypothesis and Aims

Given the increasing prevalence of glucose intolerance in pregnancy and the paucity of studies in this field, the focus of this thesis was to investigate the immediate and long-term effects of maternal glucose intolerance on offspring kidney development. It was hypothesised that offspring of pregnancies complicated by glucose intolerance would be macrosomic, yet exhibit deficits in renal development. It was further hypothesised that offspring would themselves be overweight and glucose intolerant in adulthood and exhibit perturbed renal function and morphology.

To address these hypotheses, three major studies were undertaken. This included two experimental models of glucose intolerance in pregnancy; a mouse model of pre-gestational Type 2 diabetes and a mouse model of gestational diabetes; as well as a prospective human cohort study of renal size in gestational diabetic pregnancy.

The specific aims of this thesis were:

1.

- To characterise a mouse model of pre-gestational Type 2 diabetes utilising a diet high in fat (Chapter 2).
- To assess if exposure to obesity-associated pre-gestational Type 2 diabetes affects growth and kidney development in offspring during embryogenesis and in early postnatal life (Chapter 3).
- To assess if exposure to obesity-associated pre-gestational Type 2 diabetes affects growth, glucose tolerance and body composition in adult offspring (Chapter 3).
- To assess if perturbations in kidney development have long-term consequences on renal function and morphology in adult offspring (Chapter 3).

2.

- To characterise the maternal phenotype of leptin receptor deficient (*Lepr*$^{db/+}$) mice as a model of gestational diabetes (Chapter 4).
- To assess growth and kidney development in embryonic and early postnatal offspring of *Lepr*$^{db/+}$ dams (Chapter 4).

3.

- To assess, using data from human obstetric ultrasonography, whether gestational diabetes in pregnancy affects fetal kidney size and volume as a measure of renal sufficiency (Chapter 5).

1.14 Study design

The experiments presented in Chapter 2 and 3 describe analyses of a mouse model of pre-gestational Type 2 diabetes. C57BL6/J mice were fed a high fat diet prior to and throughout pregnancy to induce obesity-related pre-gestational glucose intolerance. Characterisation of the maternal phenotype in normal fat and high fat fed mice is presented in Chapter 2. Maternal body weight, glucose intolerance, adiposity, caloric intake, and insulin levels were assessed in normal fat and high fat fed mice prior to pregnancy and in gestation and lactation. The effects of pre-gestational Type 2 diabetes on offspring growth, kidney development and adult health are presented in Chapter 3. Offspring of normal fat and high fat fed dams were collected at E15.5 (to assess ureteric branching morphogenesis and early nephron number), at E18.5 (to assess early nephron number before birth) and at PN21 (to assess final nephron endowment). Littermates were weaned on to standard laboratory chow and parameters of metabolic and renal health were assessed in adulthood at 4, 6 and 9 months of age. Measurements of

offspring body weight, glucose tolerance, body composition and renal function were assessed over time. Total glomerular volume and albumin excretion were measured in 9 month old offspring.

The experiments presented in Chapter 4 describe the analysis of a mouse model of gestational diabetes. Leptin receptor deficient mice (*Lepr*$^{db/+}$) were characterised to assess their suitability as a model of gestational diabetes. Analyses of weight gain and glucose tolerance prior to pregnancy, in late gestation and in lactation were undertaken in *Lepr*$^{db/+}$ and wild type mice. The effects of glucose intolerance on offspring growth and kidney development are also described in Chapter 4. Kidneys were collected at E18.5 and PN21 for measurement of offspring nephron number.

The experiments presented in Chapter 5 describe the analysis of human fetal kidney volume in gestational diabetic pregnancy. This chapter presents findings from a prospective cohort study using data obtained from obstetric ultrasound to assess fetal kidney volume, as a measure of renal sufficiency, in gestational diabetes. Obstetric ultrasounds were performed at 32-34 weeks gestation in women with normal glucose levels in pregnancy and women diagnosed with gestational diabetes. Fetal biometry, fetal kidney size, amniotic fluid volume, and fetal cerebral vascular resistance were measured and compared between women with and without gestational diabetes. Maternal parameters including age, ethnicity, weight and BMI and pregnancy-related data including glucose levels and treatment interventions are also presented.

CHAPTER TWO

CHARACTERISATION OF THE HIGH FAT FED MOUSE AS A MODEL OF PRE-GESTATIONAL TYPE 2 DIABETES

2.1 Introduction

Consumption of a 'Western' diet high in fat and sugar, the obesity epidemic and advances in maternal age have contributed to a growing number of pregnancies complicated by maternal glucose intolerance. Both pre-existing Type 2 diabetes and gestational diabetes have known health implications for offspring including adverse perinatal outcomes, and obesity and diabetes in adulthood. However, the effect on offspring renal development and renal health and function in postnatal life are poorly understood.

In the Western world it is estimated that over 50% of women enter pregnancy either overweight or obese (McIntyre et al., 2012, ACOG, 2013). Obesity in pregnancy is a recognised risk factor for adverse pregnancy outcomes including Caesarean section, birth weight above the 90th centile, and pre-eclampsia (Sebire et al., 2001, Avci et al., 2014). Obesity is also the strongest risk factor for the development of glucose intolerance in pregnancy as well as the development of Type 2 diabetes in non-pregnant individuals (Catalano, 2010). Almost half of all women diagnosed with gestational diabetes are overweight, obese, or extremely obese (Kim et al., 2010). The majority of women with Type 2 diabetes in pregnancy also have a BMI greater than 25 kg/m^2 (AIHW, 2008).

Pre-existing Type 2 diabetes is reported in over 2% of pregnancies. However this is likely an underestimate as 14% of women of reproductive age exhibit undiagnosed impaired glucose tolerance or pre-diabetes (Dunstan et al., 2002, Razzaghi et al., 2013, CDC, 2014). This may potentially lead to a significant number of women with unrecognised pre-gestational diabetes, since people with pre-diabetes are at high risk of developing Type 2 diabetes. Conventional screening for gestational diabetes occurs between 24-28 weeks gestation and will identify pregnancy-related glucose handling abnormalities (classic gestational diabetes) in 5-17% of pregnancies (Templeton and Pieris-Caldwell, 2008, IADPSG Consensus Panel, 2010, Moses et al., 2011, DeSisto et al., 2014). Screening will also identify an unknown number of women with previously undiagnosed pre-gestational Type 2 diabetes that will be classified with gestational diabetes.

In recognition of the substantial and increasing number of women with pre-existing glucose intolerance, new guidelines have been developed (IADPSG Consensus Panel, 2010). It is now recommended to screen women earlier in pregnancy at first antenatal visit if they have previous gestational diabetes, previous macrosomia or BMI > 35 kg/m^2 (ADIPS, 2014). Unfortunately, these recommendations are not universally implemented.

Hyperglycaemia is known to adversely affect kidney development. Numerous human studies have identified an increased risk of congenital anomalies of the kidneys and urinary tract in children born to diabetic mothers (Sheffield et al., 2002, Kamdem et al., 2005, Nielsen et al., 2005, Shnorhavorian et

al., 2011, Hsu et al., 2014). In addition, several rodent studies have reported a deficit in offspring nephron number (Amri et al., 1999, Tran et al., 2008, Hokke et al., 2013). These animal studies are primarily based on the protocol of STZ-induced diabetes as a model of Type 1 diabetes, which typically elicits severe and persistent fasting hyperglycaemia in the dam as well as profound growth restriction in the offspring. While these studies contribute to our fundamental understanding of how very high glucose concentrations affect the developing kidney, they are limited as they only model the condition seen in a small subset of human pregnancies with poorly controlled Type 1 diabetes complicated by pre-eclampsia and/or vascular disease. Furthermore, several of these publications do not model pre-gestational diabetes since hyperglycaemia was induced after the commencement of kidney formation (Amri et al., 1999, Tran et al., 2008).

High fat feeding has been used extensively in rodents to model Type 2 diabetes (Surwit et al., 1988, Collins et al., 2004, Winzell and Ahrén, 2004). High fat fed rodents develop obesity, insulin resistance, glucose intolerance and β cell dysfunction. Differences in dietary composition, age of onset of high fat feeding, length of feeding, sex and rodent strain contribute to variability in the metabolic perturbations observed across these studies (Lai et al., 2014). To a lesser extent, high fat or 'Western' diets have been used in pregnancy to model pre-gestational Type 2 diabetes (Holemans et al., 2004, Samuelsson et al., 2008, Liang et al., 2009a, Nivoit et al., 2009, Li et al., 2013). This model shares similar features with the human condition - pre-gestational obesity, glucose intolerance and hyperinsulinaemia, as well as reduced gestational weight gain and macrosomic offspring - but has yet to be applied in studies investigating offspring kidney development and renal function in the setting of maternal diabetes.

Despite pre-gestational Type 2 diabetes being an increasingly common complication of pregnancy, to date no rodent study has investigated how pre-existing maternal glucose intolerance affects the developing kidney. High fat feeding presents a suitable rodent model of obesity-associated pre-gestational Type 2 diabetes in which to investigate offspring kidney development and subsequent renal function. In the studies described in this chapter, female mice were fed a high fat diet prior to and throughout pregnancy to establish the maternal phenotype. The phenotype of offspring exposed to high fat fed dams is presented in Chapter 3.

2.2 Aim

- To characterise a mouse model of pre-gestational Type 2 diabetes utilising a diet high in fat.

2.3 Hypothesis

- Mice fed a high fat diet will exhibit increased weight gain and impaired glucose tolerance prior to and throughout pregnancy modelling pre-gestational Type 2 diabetes.

2.4 Methods

2.4.1 Animal husbandry

All animal handling and experimental protocols were approved by the Animal Ethics Committee of Monash University (MARP/2011/122) and conformed to the guidelines of the National Health and Medical Research Council of Australia. Female C57BL6/J mice were purchased from Monash Animal Research Platform at 5 weeks of age. Mice were housed in groups of five in Optiplex cages with sawdust bedding in a controlled environment (20-22°C, 11-45% humidity) with a 12/12-hour light-dark cycle. Mice had *ad libitum* access to water and rodent diet.

2.4.2 Diet

Female mice were placed on either a high fat diet (21% fat w/w; SF00-219, Specialty Feeds semi-pure rodent diet; Specialty Feeds, Glen Forrest, WA, Australia; see **Table 2.1** for nutritional parameters and ingredients) or matched normal fat diet (6% fat w/w; SF04-057, Specialty Feeds semi-pure rodent diet; Specialty Feeds, Glen Forrest, WA, Australia, see **Table 2.1** for nutritional parameters and ingredients). The SF00-219 high fat diet was specifically chosen as it is designed to mimic a 'Western fast food diet' and is comparable to the Harlan Teklad TD88137 diet which has been previously used in rodent studies of diet-induced obesity and diabetes (Winzell and Ahrén, 2004, Buettner et al., 2007, Wang and Liao, 2012). Due to concerns regarding disparities in protein, vitamin and mineral content between standard chow and high fat formulations, a matched diet was fed to control mice. This matched diet only differed from the high fat diet in lipid content (21% vs 6%, source changed from Ghee to Canola oil to maintain requirements of some individual fatty acids), starch content (increased to match the removal of Ghee), and had no added cholesterol. Five-week old mice were placed on their respective diet for 6 weeks prior to pregnancy and throughout gestation and lactation until PN21, a protocol used previously by Samuelsson et al. (2008).

Table 2.1 Nutritional parameters and ingredients of the 21% high fat diet SF00-219 and the 6% normal fat matched diet SF04-057

	21% Fat, 0.15% Cholesterol Semi-Pure Rodent Diet SF00-219	6% Fat Semi-Pure Rodent Diet SF04-057
Calculated Nutritional Parameters		
Protein	19%	19%
Fat	21%	6%
Crude Fibre	4.7%	4.7%
Adequate Dietary Fibre	4.7%	4.7%
Digestible Energy	19.4 MJ/kg	16.1 MJ/kg
% Total digestible energy from lipids	40%	14%
% Total digestible energy from protein	17%	21%
Ingredients (g/kg)		
Casein (Acid)	195	195
Sucrose	341	341
Fat source	210 (Ghee)	60 (Canola oil)
Cellulose	50	50
Wheat Starch	145	306
DL Methionine	3.0	3.0
Calcium Carbonate	17.1	17.1
Sodium Chloride	2.6	2.6
AIN93 Trace Minerals	1.4	1.4
Potassium Citrate	2.6	2.5
Potassium Dihydrogen Phosphate	6.9	6.9
Potassium Sulphate	1.6	1.6
Choline Chloride (75%)	2.5	2.5
SF00-219 Vitamins	10	10
Cholesterol	1.5	0
Oxicap E2	0.04	0.04
Calculated Fatty Acid Composition (%)		
Saturated Fats C12:0 or less	1.8	No data
Total Mono Unsaturated Fats	No data	3.43
Total Poly Unsaturated Fats	No data	2.15
Total Saturated Fats	No data	0.43
Myristic Acid 14:0	2.6	Trace
Palmitic Acid 16:0	7.0	0.3
Stearic Acid 18:0	2.4	0.1
Palmitoleic Acid 16:1	0.4	Trace
Oleic Acid 18:1	5.5	3.3
Linoleic Acid 18:2 n6	0.4	1.3
a Linolenic Acid 18:3 n3	0.20	0.9
Arachadonic Acid 20:4 n6	Trace	No data
EPA 20:5 n3	No data	No data
DHA 22:6 n3	No data	No data
Total n3	0.35	0.9
Total n6	0.41	1.3
Cholesterol	0.15	0

2.4.3 Weight and food intake pre-pregnancy

Mice were weighed weekly prior to pregnancy. Food intake was recorded in a cohort of mice throughout the 6 week pre-pregnancy period. To measure food intake, mice were housed in pairs. Every 2-3 days the weight of food removed from the food hopper and the weight of bedding and food left on the base of the cage was recorded and used to calculate food consumption per mouse. Total food intake was calculated for the 6 week pre-pregnancy period. Total energy intake was calculated for each diet group based on the digestible energy values of 19.4 MJ/kg for the high fat diet and 16.1 MJ/kg for the normal fat diet.

2.4.4 Glucose tolerance test

As a preliminary assessment, a small cohort of mice underwent a glucose tolerance test (GTT) at 3 weeks post diet to assess for early glucose intolerance in high fat fed mice. Five weeks post diet, GTTs were performed in all mice. To measure glucose tolerance, mice were fasted for 6 hours and injected intraperitoneally with D-glucose (Sigma Aldrich, Castle Hill, NSW, Australia) in sterile saline (2 g/kg body weight). Blood was sampled from the tail vein prior to and at 30, 60, 90 and 120 minutes after injection and blood glucose concentration was measured immediately using a glucometer (Accu-chek Mobile Blood Glucose Monitor; Roche Diagnostics, Castle Hill, NSW, Australia). The tail was wiped with 80% ethanol and the lateral tail vein pricked with a sterile 26 gauge needle. Approximately 5 µl of blood from the tail tip was required for each measurement of blood glucose concentration. Consecutive blood samples at 30 minute intervals were taken following removal of the clot. Based on the glucose tolerance curve, a glucose Area Under the Curve (AUC) value was calculated for each mouse.

2.4.4.1 Glucose tolerance test in pregnancy and at weaning

GTTs were also performed in cohorts of pregnant dams at E15.5, E18.5 and postnatally at PN21.

2.4.5 Body composition

Dual energy X-ray absorptiometry (DEXA) is considered the gold-standard method for longitudinal analysis of body composition. DEXA measures body composition and bone density by exposing the subject to a small source of X-rays at high and low energies. Energy counts are detected and a high-resolution digital image is obtained. Using PIXImus software (2.10; GE/Lunar) the ratio of attenuation of the two energies provides quantitative data on bone mineral density, bone mineral content, bone area, lean tissue mass, fat tissue mass, total tissue mass and percentage fat.

Body composition was measured in a cohort of mice 5 weeks post diet by DEXA scan (PIXImus2; Lunar, Madison, WI, USA). Each mouse was anaesthetised by exposure to 2.5% Isoflurane-oxygen gas (Isorane; Baxter, Old Toongabbie, NSW, Australia), administered via a nose cone. Mice were placed on

the scanner bed in the prone position, with the limbs slightly outstretched, tail curled around the side of the body, and the spine in a straight line. One scan per mouse was performed and analysed with PIXImus software. Each scan took approximately 5 minutes. The head of each animal was excluded from analysis using a manual region of interest. The DEXA scanner was calibrated on each day of testing using PIXImus software and an aluminium/Lucite phantom supplied by the manufacturer (corresponding to bone mineral density = 0.0592 g/cm^2 and 12.5% fat).

2.4.6 Mating protocol

Six weeks post diet, female mice of approximately 11 weeks of age were mated overnight with male C57BL6/J mice. The following morning females were checked for the presence of a vaginal seminal plug. The presence of a vaginal plug confirmed that mating had taken place and indicated E0.5 of pregnancy. Females were maintained on their respective diets throughout the mating period. To circumvent the confounding effect of male high fat feeding, male studs were interchanged between cages of high fat fed and normal fat fed females every 48 hours.

2.4.7 Weight and food intake during pregnancy and lactation

Maternal weight was recorded at time of positive vaginal plug and throughout pregnancy and lactation. Gestational weight gain was calculated by subtracting the weight of dams at E0.5 from their weight at E18.5. Food intake was measured throughout pregnancy in a cohort of dams housed individually or in pairs according to diet. Food consumption was calculated as described above (**Section 2.4.3**). Total food intake was calculated for the period of E0.5 to E18.5.

2.4.8 Plasma analyses

Insulin and leptin protein content were quantified in maternal plasma samples by enzyme-linked immunosorbant assay (ELISA) at E18.5.

Dams were anaesthetised using the inhalation anaesthetic Isoflurane in an induction chamber at E18.5. Following absence of pedal reflex an incision was made along the thoracic wall and through the diaphragm and ribcage to expose the heart. Mice were exsanguinated by left ventricular puncture using a 26-gauge needle. Approximately 500-1000 µl of blood was collected into an Eppendorf tube containing ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich, Castle Hill, NSW, Australia) to prevent coagulation. Blood was centrifuged for 10 minutes at 4°C at 3000 rpm. Maternal plasma was aspirated from packed cells and stored at -80°C until required.

2.4.8.1 Plasma Insulin

To measure plasma insulin, an ultra-sensitive mouse insulin ELISA kit (#90080, Crystal Chem, Downers Grove, IL, USA) was used. This assay employs a guinea pig anti-insulin antibody coated on a 96-well

microplate. To each well, 5 µl of plasma sample or mouse insulin standard were pipetted with 95 µl of provided sample diluent. Samples were incubated for 2 hours at 4°C to allow mouse insulin to bind to the immobilised antibody. Wells were washed five times with the provided wash buffer to remove unbound material. 100 µl of horseradish peroxidase (HRP)-conjugated anti-insulin antibody was dispensed in each well and incubated for 30 minutes at room temperature. Wells were subsequently washed seven times before 100 µl of 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution was added. TMB is a chromogenic substrate that reacts with HRP causing a blue colour to develop in proportion to the amount of bound insulin. Following a 40 minute incubation at room temperature, the enzyme reaction was stopped by adding 100 µl of 1N sulphuric acid changing the colour from blue to yellow.

Colour intensity was measured on a microplate reader (xMark Microplate Absorbance Spectrophotometer, Bio-Rad, Hercules, CA, USA) using dual wavelengths (A_{450} values measured and A_{630} values subtracted). Insulin concentrations were calculated from a 4-parameter fit standard curve using Microplate Manager software (version 6.1, Bio-Rad, Hercules, CA, USA).

All samples were run in duplicate and a mean concentration value used in analysis. The coefficient of variation was 7.0%.

2.4.8.2 Plasma Leptin

Plasma leptin, was measured using a mouse leptin ELISA kit (#90030; Crystal Chem, Downers Grove, IL, USA). A 96-well microplate coated with rabbit anti-leptin antibody was washed twice with the provided wash buffer. 45 µl of sample diluent, 50 µl of guinea pig anti-mouse leptin serum, and 5 µl of plasma sample or mouse leptin standards were dispensed into each well. The microplate was incubated overnight at 4°C to allow the simultaneous binding of leptin in the sample to rabbit anti-leptin antibody and the anti-leptin IgG of the guinea pig anti-serum. Unbound material was removed by washing five times. 100 µl of HRP-conjugated anti-guinea pig IgG antibody was dispensed in each well and allowed to bind to the immobilised complex for 3 hours at 4°C. Excess HRP-conjugated antibody was removed by washing with wash buffer seven times. 100µl of TMB substrate solution was subsequently dispensed in each well and allowed to react with bound HRP-conjugated antibody for 30 minutes at room temperature. The enzyme reaction was stopped by adding 100 µl of 1N sulphuric acid.

Colour intensity was measured on a microplate reader using dual wavelengths (A_{450} values measured and A_{630} values subtracted). Leptin concentrations were calculated from a 4-parameter fit standard curve using Microplate Manager software.

All samples were run in duplicate and a mean concentration value used in analysis. The coefficient of variation was 4.7%.

2.4.9 Statistical analysis

Data were analysed using SPSS statistics software (version 20, SPSS Inc., USA) and GraphPad Prism software (version 6, GraphPad Software, Inc., USA). Weight, weight gain, food intake, body composition and plasma insulin and leptin were analysed by independent samples t-test. Weight prior to pregnancy and GTTs were analysed by repeated measures ANOVA. A glucose AUC value of the GTT was calculated using GraphPad and analysed by independent samples t-test. Spearman's rank coefficient with Bonferroni correction was performed using STATA data analysis and statistical software (version 8.0, Stata Corporation, USA). Data were tested for normality using a D'Agostino-Pearson omnibus normality test. Data are presented as mean ± standard error of the mean (SEM). $P<0.05$ was considered statistically significant.

2.5 Results

2.5.1 The high fat fed mouse is a model of obesity-associated pre-gestational glucose intolerance

2.5.1.1 Preliminary assessment: Three weeks of high fat feeding is insufficient to induce glucose intolerance

A small cohort of mice (normal fat diet (NFD), *n*=6; high fat diet (HFD), *n*=7) underwent a GTT after 3 weeks into the 6 week pre-pregnancy diet period. While mice fed a high fat diet were significantly heavier than mice fed the normal diet after 3 weeks (NFD 19.9 ± 0.6 g, HFD 21.9 ± 0.5 g; *P*=0.031), there was no difference in glucose profile (P_{diet}=0.485; **Figure 2.1A**) or glucose AUC (NFD 1496.1 ± 86.1 mmol/l.min, HFD 1599.5 ± 78.6 mmol/l.min; *P*=0.397).

2.5.1.2 Preliminary assessment: Five weeks of high fat feeding induces glucose intolerance

Five weeks post diet, repeat GTTs were performed in the same cohort of mice. Compared to mice fed a normal fat diet, mice fed a high fat diet for 5 weeks exhibited increased weight (NFD 21.3 ± 0.6 g, HFD 25.0 ± 0.9 g; *P*=0.006) and a significantly elevated glucose profile (*P*=0.006; **Figure 2.1B**). Glucose AUC was also greater after 5 weeks of high fat feeding (NFD 1369.2 ± 79.8 mmol/l.min, HFD 1674.5 ± 47.3 mmol/l.min; *P*=0.006).

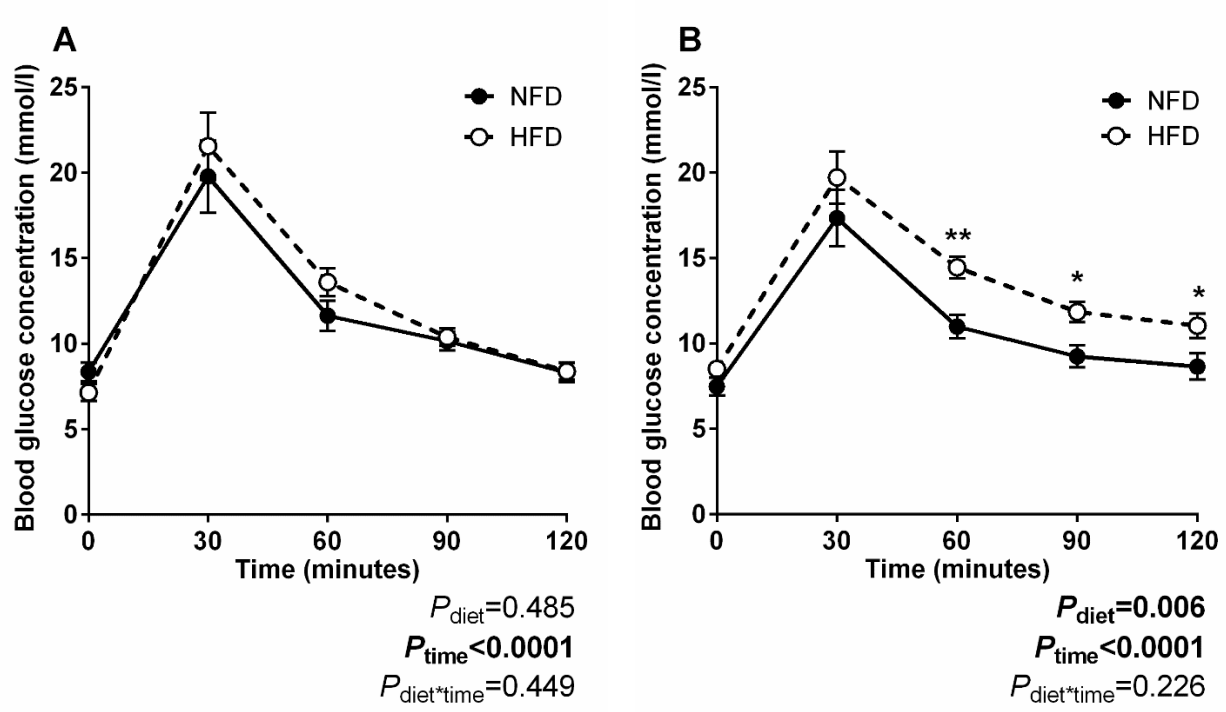


Figure 2.1 Preliminary glucose tolerance tests at 3 and 5 weeks post diet
Mean GTT values in NFD and HFD fed mice **A** 3 weeks and **B** 5 weeks post diet. GTT analysed by repeated measures ANOVA for maternal diet and time and post-hoc t-test. Values are mean ± SEM. *n*(NFD)=6, *n*(HFD)=7. **P*<0.05, ***P*<0.01 refer to differences between the two groups at the each time point.

2.5.1.3 Obesity-associated pre-gestational glucose intolerance in high fat fed mice

Subsequently, all mice (*n*(NFD)=86, *n*(HFD)=93) were assessed for glucose intolerance 5 weeks post diet, and mated after 6 weeks. Compared to mice fed a normal fat diet, mice on a high fat diet were 12% heavier than control mice following 5 weeks of high fat feeding (NFD 22.9 ± 0.2 g, HFD 25.7 ± 0.3 g; *P*<0.0001; **Figure 2.2**) and had a significantly elevated glucose profile (*P*<0.0001; **Figure 2.3**). Post-hoc analysis revealed no change in fasting glucose (NFD 7.6 ± 0.2 mmol/l, HFD 8.1 ± 0.2 mmol/l; *P*=0.082) yet significantly greater blood glucose concentrations at each time point following a glucose load (*P*<0.0001 for 30, 60, 90 and 120 minutes). Glucose AUC was statistically greater by 20% in mice fed a high fat diet (NFD 1355.1 ± 26.2 mmol/l.min, HFD 1631.9 ± 28.1 mmol/l.min; *P*<0.0001; **Figure 2.4**).

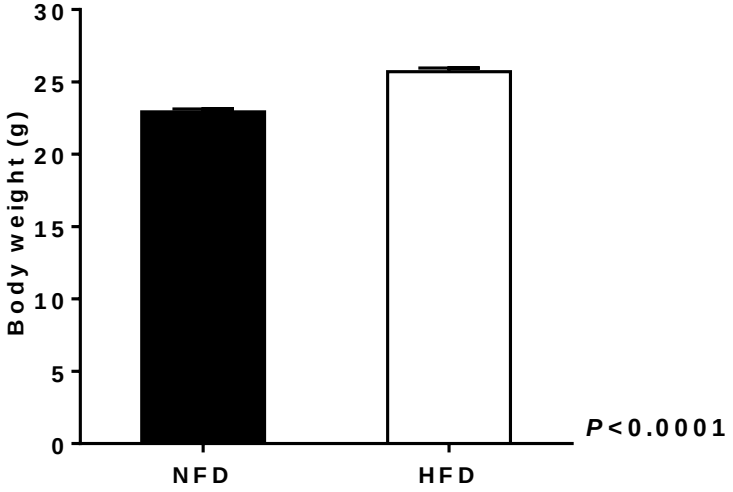


Figure 2.2 Body weight 5 weeks post diet
Mean body weight for NFD and HFD fed mice 5 weeks post diet. Analysed by independent samples t-test. Values are mean ± SEM. *n*(NFD)=86, *n*(HFD)=93.

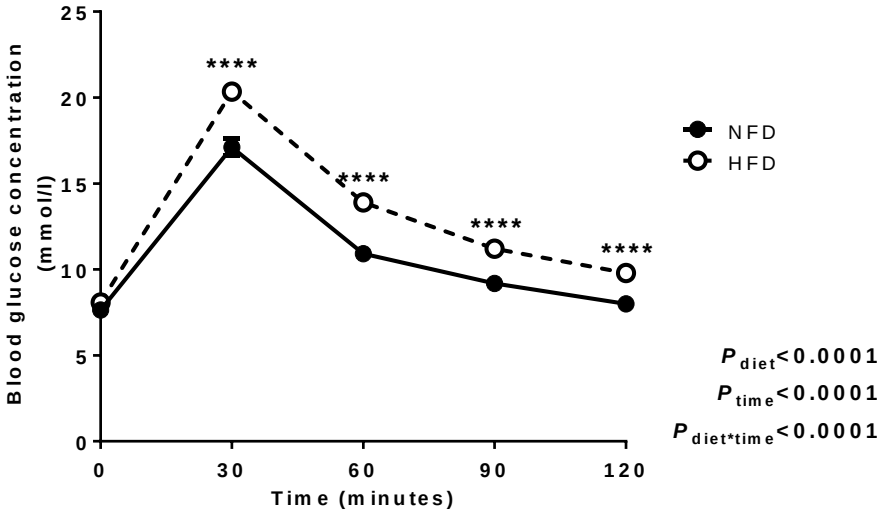


Figure 2.3 Glucose tolerance tests 5 weeks post diet

Mean GTT curves for NFD and HFD fed mice 5 weeks post diet. GTT analysed by repeated measures ANOVA for maternal diet and time and post-hoc t-test. Values are mean ± SEM. *n*(NFD)=86, *n*(HFD)=93. **** *P*<0.0001 refers to difference between the two groups at the each time point.

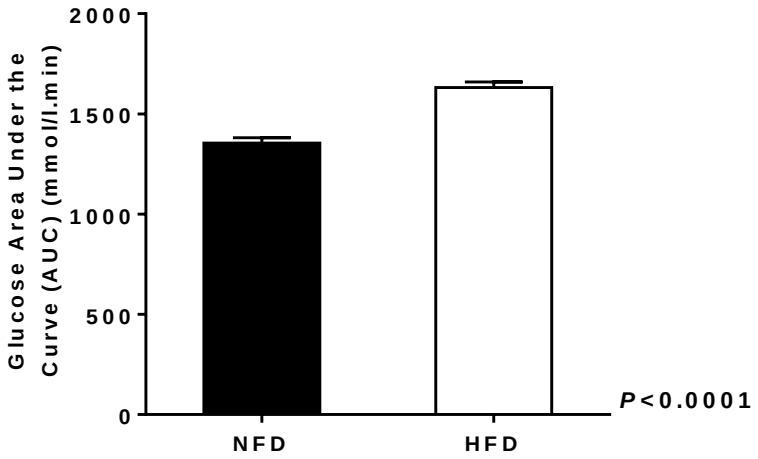


Figure 2.4 Glucose AUC 5 weeks post diet

Glucose AUC for NFD and HFD fed mice 5 weeks post diet. Analysis by independent samples t-test. Values are mean ± SEM. *n*(NFD)=86, *n*(HFD)=93.

2.5.1.4 Defining the glucose tolerant and glucose intolerant mouse

As presented in **Figure 2.3** and **Figure 2.4**, mice fed a high fat diet for 5 weeks had, on average, a significantly greater glucose profile than mice fed a normal fat diet. The glucose AUC of each mouse was assessed to identify the variability in glucose tolerance in both normal fat and high fat fed mice. These individual values are illustrated in **Figure 2.5**.

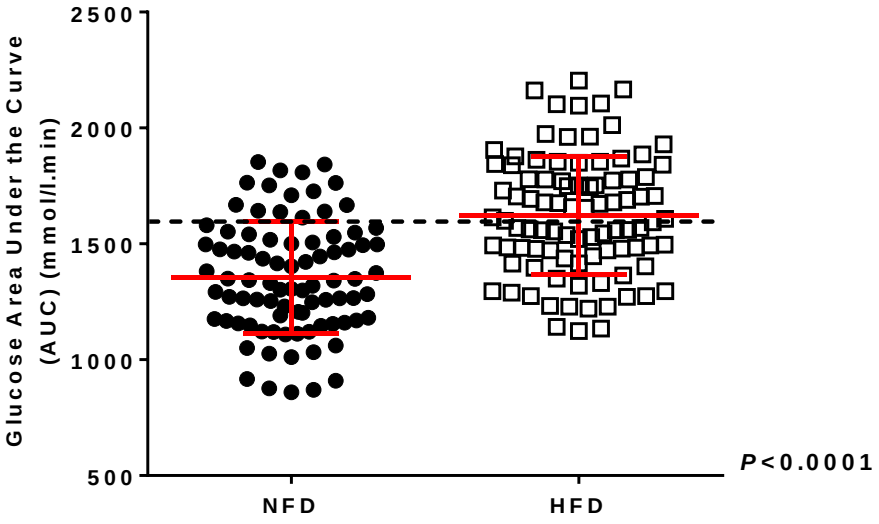


Figure 2.5 Individual glucose AUC values 5 weeks post diet
Individual glucose AUC values for mice fed a NFD or HFD at 5 weeks post diet. Mean values and SD are indicated in red. *n*(NFD)=86, *n*(HFD)=93.

Due to the heterogeneity in glucose AUC of mice fed a normal or high fat diet, specific criteria were applied to categorise mice as either 'glucose tolerant' or 'glucose intolerant'. Glucose AUC values, derived from the GTT performed at 5 weeks post diet, were analysed for all mice fed a normal fat diet. Normal fat fed mice that fell within one standard deviation of the mean were classified with normal glucose tolerance (pre-pregnancy glucose AUC value between 1112-1598 mmol/l.min). High fat fed mice with a glucose AUC greater than one standard deviation above the mean AUC value of normal fat fed mice were classified with impaired glucose tolerance (pre-pregnancy glucose AUC > 1598 mmol/l.min). A visual representation of the selection criteria is presented in **Figure 2.6**. Throughout the remainder of this chapter and Chapter 3, data are presented for mice that fit this criteria. Normal fat fed mice with normal glucose tolerance will be referred to as NFD NGT, and high fat fed mice with impaired glucose tolerance will be referred to as HFD IGT. Mice (and their offspring) with glucose AUC values outside the specified criteria were excluded from analysis unless otherwise stated.

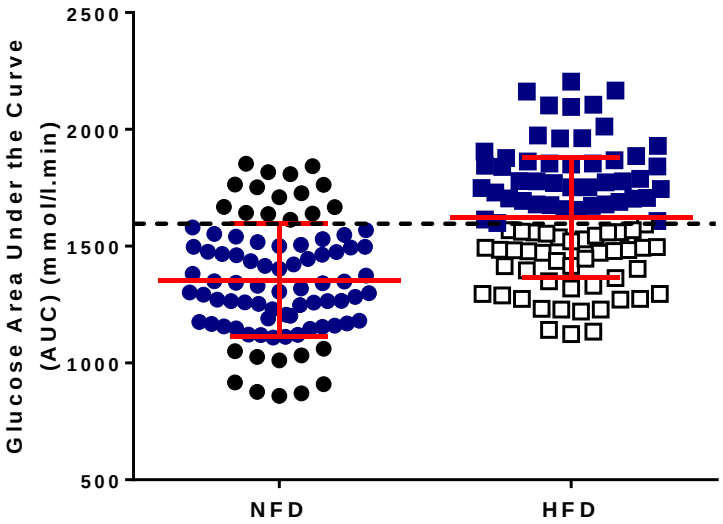


Figure 2.6 Individual glucose AUC values 5 weeks post diet, highlighting mice that met the selection criteria

Individual glucose AUC values for mice fed a NFD or HFD at 5 weeks post diet. NFD NGT and HFD IGT mice that met the selection criteria are highlighted in blue. Dashed line indicates cut-off criteria used to select HFD IGT dams (pre-pregnancy glucose AUC > 1598 mmol/l.min). Red bars indicate mean ± SD.

Data from GTTs for mice that met the selection criteria specified above are presented in **Figure 2.7**. Compared with NFD NGT mice, HFD IGT mice had a significantly elevated fasting blood glucose concentration (NFD NGT 7.7 ± 0.2 mmol/l, HFD IGT 8.6 ± 0.2 mmol/l; *P*=0.003) and exhibited a 44% higher peak glucose concentration 30 minutes after a glucose load (NFD NGT 16.3 ± 0.4 mmol/l, HFD IGT 23.5 ± 0.5 mmol/l; *P*<0.0001). Blood glucose concentrations were also elevated at 60, 90 and 120 minutes (*P*<0.0001). Glucose AUC was 39% higher in HFD IGT mice 5 weeks post diet (NFD NGT 1323.1 ± 18.64 mmol/l.min, HFD IGT 1841.5 ± 26.5 mmol/l.min; *P*<0.0001).

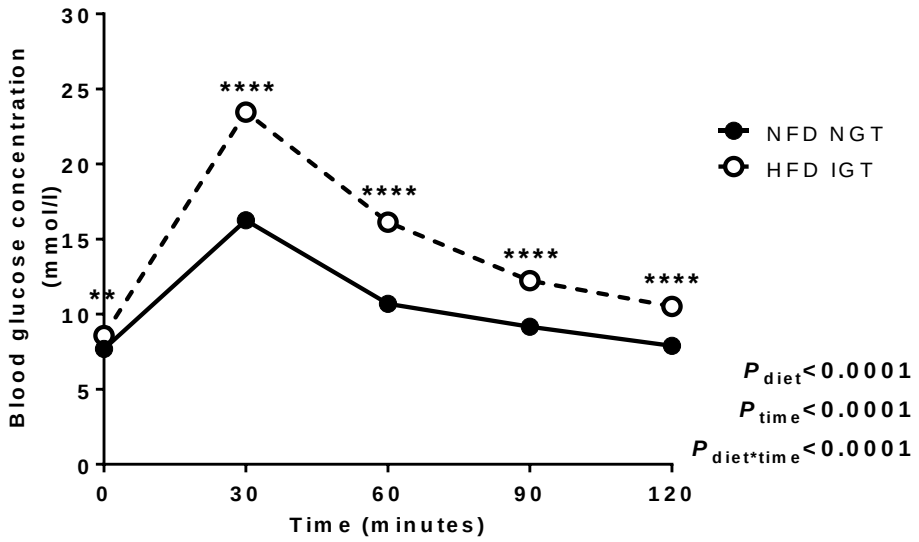


Figure 2.7 Glucose tolerance tests 5 weeks post diet in mice that met the selection criteria
Mean GTT curves for NFD NGT and HFD IGT mice 5 weeks post diet. GTT analysed by repeated measures ANOVA for maternal diet and time and post-hoc t-test. *P*<0.0001. *n*(NFD NGT)=62, *n*(HFD IGT)=48. Values are mean ± SEM. ** *P*<0.01 and **** *P*<0.0001 refer to the difference between the two groups at each time point.

2.5.1.5 Predicting glucose intolerance in high fat fed mice

To assess if body weight could predict glucose intolerance in high fat fed mice, body weights were compared between high fat fed mice with normal glucose tolerance (HFD NGT; pre-pregnancy glucose AUC < 1598 mmol/l.min) and HFD IGT mice prior to pregnancy. There was no difference in body weight (HFD NGT 25.41 ± 0.38 g, HFD IGT 25.98 ± 0.34 g; *P*=0.537; **Figure 2.8A**) or weight gain (HFD NGT 7.79 ± 0.30 g, HFD IGT 8.07 ± 0.33 g; *P*=0.284; **Figure 2.8B**) after 6 weeks of high fat diet in mice that did and did not exhibit glucose intolerance.

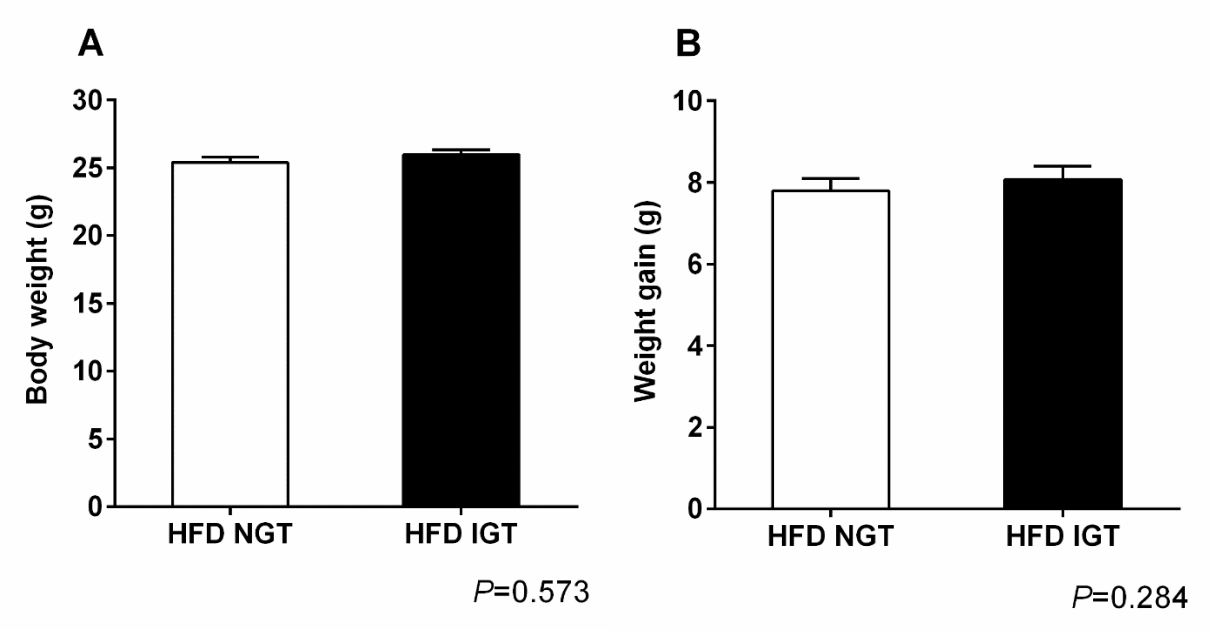


Figure 2.8 Body weight and weight gain prior to pregnancy
A Body weight and **B** weight gain after 6 weeks of high fat feeding. Analysis by independent samples t-test. Values are mean ± SEM. *n*(HFD NGT)=462 *n*(HFD IGT)=48.

2.5.1.6 Increased body weight and energy intake prior to pregnancy

Compared with NFD NGT mice, HFD IGT mice exhibited increased body weight from week one to week six of the diet protocol (*P*<0.0001*;* **Figure 2.9A**). Following 6 weeks on diet, HFD IGT mice were 15% heavier than NFD NGT mice (NFD NGT 22.6 ± 0.27 g, HFD IGT 26.0 ± 0.3 g; *P*<0.0001). Total weight gain was 67% greater in HFD IGT mice over the 6 week pre-pregnancy period (NFD NGT 4.87 ± 0.20 g, HFD IGT 8.07 ± 0.33 g; *P*<0.0001; **Figure 2.9B**).

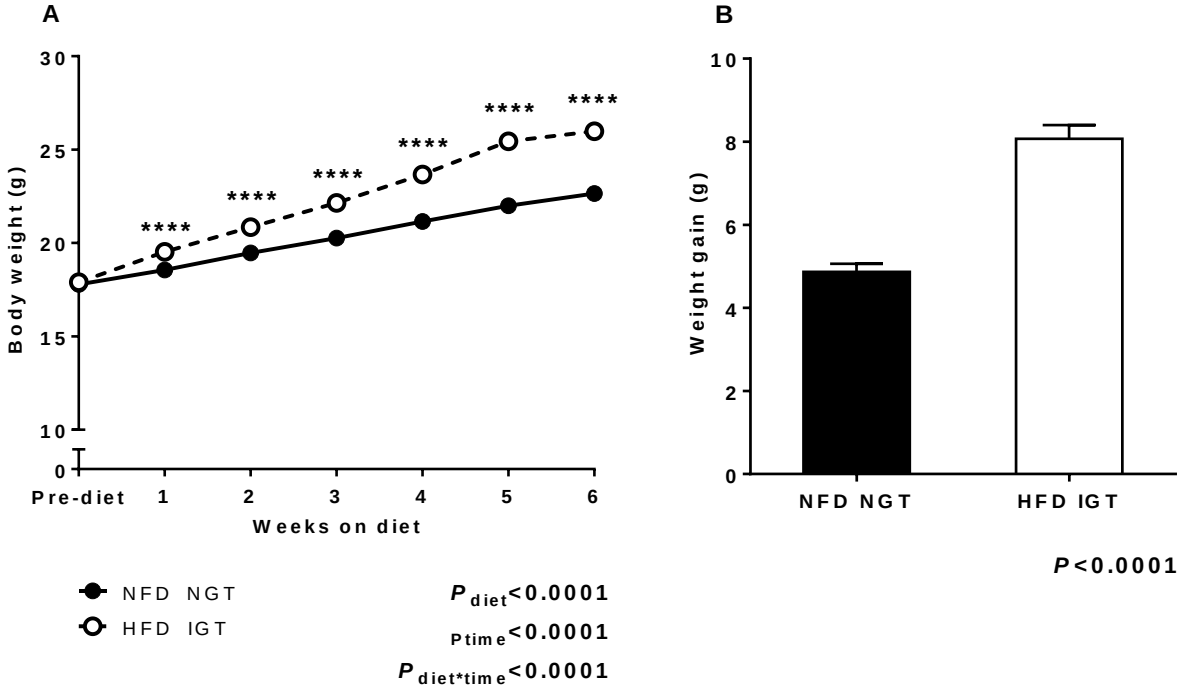


Figure 2.9 Body weight during the 6 week diet protocol
A Body weight across the 6 week diet protocol in NFD NGT mice and HFD IGT mice. **B** Total weight gain after 6 weeks on the diet in NFD NGT and HFD IGT mice. Analysis by repeated measures ANOVA and independent samples t-test. Values are mean ± SEM. *n*(NFD NGT)=62, *n*(HFD IGT)=48. ****P<0.0001 refers to the difference between the two groups at each time point.

Food intake during the 6 week pre-pregnancy period was measured in a cohort of mice. There was no difference in the total weight of food consumed by NFD NGT or HFD IGT mice (NFD NGT 2.49 ± 0.13 g/day, HFD IGT 2.37 ± 0.3 g/day; P=0.394; **Figure 2.10A**). Due to the greater digestible energy, mice fed a high fat diet had a significantly greater total energy intake than mice fed a normal fat diet (NFD NGT 40.08 ± 2.01 kJ/day, HFD IGT 46.00 ± 0.63 kJ/day; P=0.031; **Figure 2.10B**).

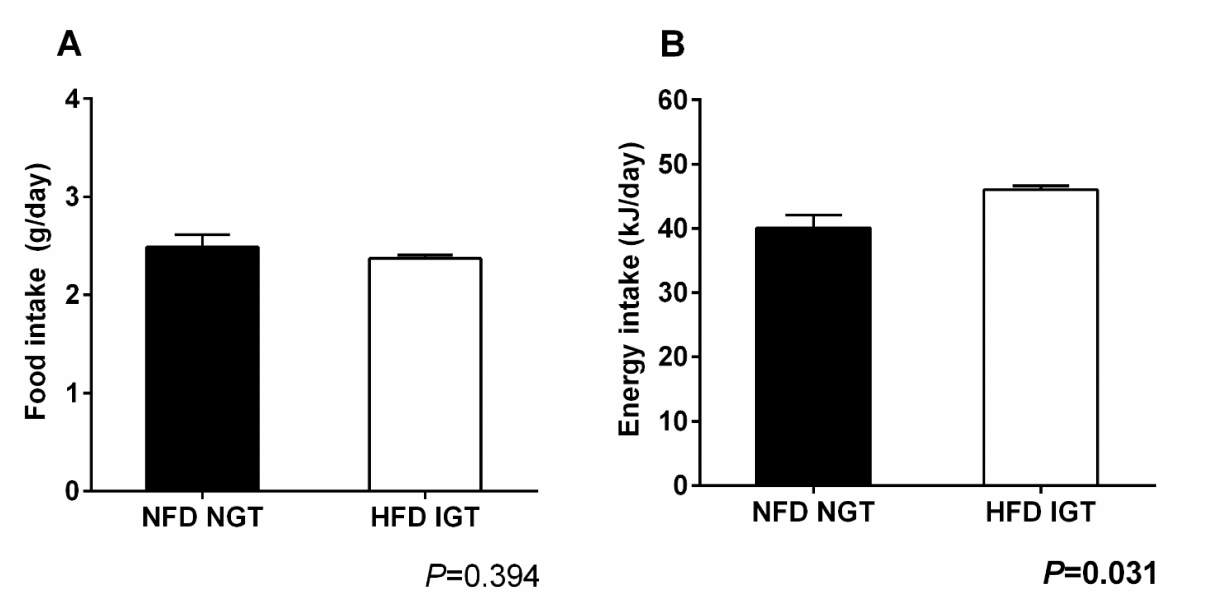


Figure 2.10 Food intake and energy intake during the 6 week diet protocol
A Daily food intake and **B** daily energy intake in NFD NGT mice and HFD IGT mice in the 6 week pre-pregnancy period. Analysis by independent samples t-test. Values are mean ± SEM. *n*(NFD NGT)=6, *n*(HFD IGT)=6.

2.5.1.7 Increased fat content prior to pregnancy

DEXA scans revealed a significantly greater lean tissue mass (*P*=0.02), fat tissue mass (*P*=0.0005), total tissue mass (*P*<0.0001) and percentage body fat (28% increase, *P*=0.002) in HFD IGT mice compared with NFD NGT mice after 5 weeks on diet. Body composition parameters are outlined in **Table 2.2**. Representative DEXA scans from NFD NGT HFD IGT mice are shown in **Figure 2.11**.

Table 2.2 Body composition 5 weeks post diet

	NFD NGT (*n*=15)	**HFD IGT** (*n*=20)	***P***
Lean tissue, g	17.2 ± 0.2	18.0 ± 0.2	**0.02**
Fat tissue, g	5.4 ± 0.3	8.1 ± 0.5	**0.0005**
Total tissue, g	22.6 ± 0.4	26.1 ± 0.6	**<0.0001**
Percentage fat, %	23.7 ± 1.1	30.4 ± 1.5	**0.002**

Analysis by independent samples t-test. Values are mean ± SEM.

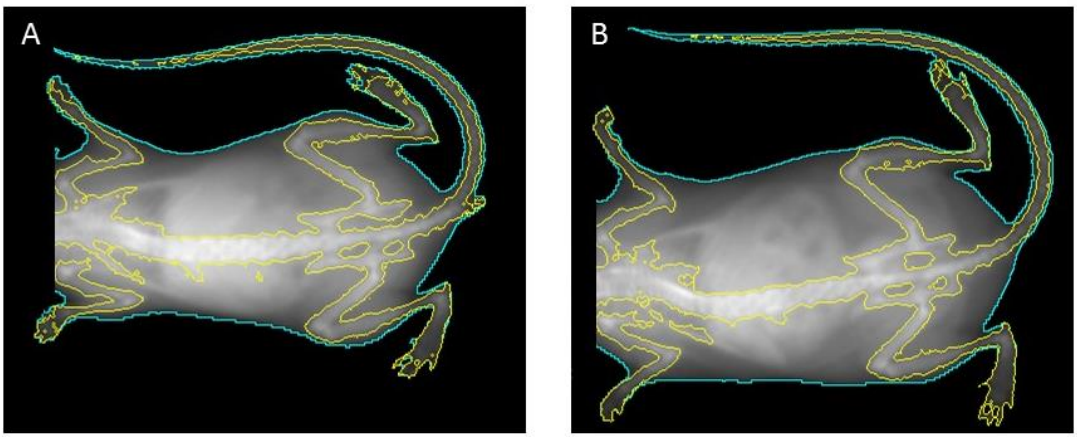


Figure 2.11 Representative DEXA scan 5 weeks post diet
Representative DEXA scans in **A** a NFD NGT mouse and **B** a HFD IGT mouse after 5 weeks on diet.

2.5.2 Glucose tolerance and body weight are similar in pregnancy

2.5.2.1 Greater body weight but reduced gestational weight gain in pregnancy

Maternal body weights are presented in **Table 2.3**. HFD IGT mice were significantly heavier than NFD NGT mice at the day of positive vaginal plug E0.5 (10% weight difference, *P*=0.0004) and at E15.5 (8% weight difference, *P*=0.004). However, at E18.5 there was no difference in weight between NFD NGT and HFD IGT dams, indicating a reduced rate of weight gain in late pregnancy in HFD IGT dams. Overall, gestational weight gain measured between E0.5 and E18.5 was 14% lower in HFD IGT dams (NFD NGT 15.7 ± 0.6 g, *n*=21; HFD IGT 13.6 ± 0.4 g, *n*=21; *P*=0.004). Maternal body weight at weaning (PN21) was similar in the two groups.

Table 2.3 Maternal body weight in pregnancy and at weaning

Body weight (g)	**NFD NGT** (*n*)	**HFD IGT** (*n*)	***P***
E0.5	24.1 ± 0.4 (46)	26.5 ± 0.5 (34)	**0.0004**
E15.5	35.1 ± 0.6 (13)	38.0 ± 0.7 (13)	**0.004**
E18.5	39.8 ± 0.9 (21)	39.8 ± 0.7 (21)	0.995
PN21	31.1 ± 0.7 (15)	32.0 ± 1.2 (10)	0.564

Analysis by independent samples t-test. Values are mean ± SEM.

2.5.2.2 Increased energy intake in pregnancy

Food intake was measured in a cohort of pregnant dams between E0.5 and E18.5. As observed prior to pregnancy, there was no difference between the two groups in the weight of food consumed (NFD NGT 2.33 ± 0.08 g/day, HFD IGT 2.63 ± 0.26 g/day; *P*=0.266; **Figure 2.12A**), however, HFD IGT dams had a 36% greater energy intake per day during pregnancy (NFD NGT 37.57 ± 1.32 kJ/day, HFD IGT 50.92 ± 4.90 kJ/day; *P*=0.018; **Figure 2.12B**).

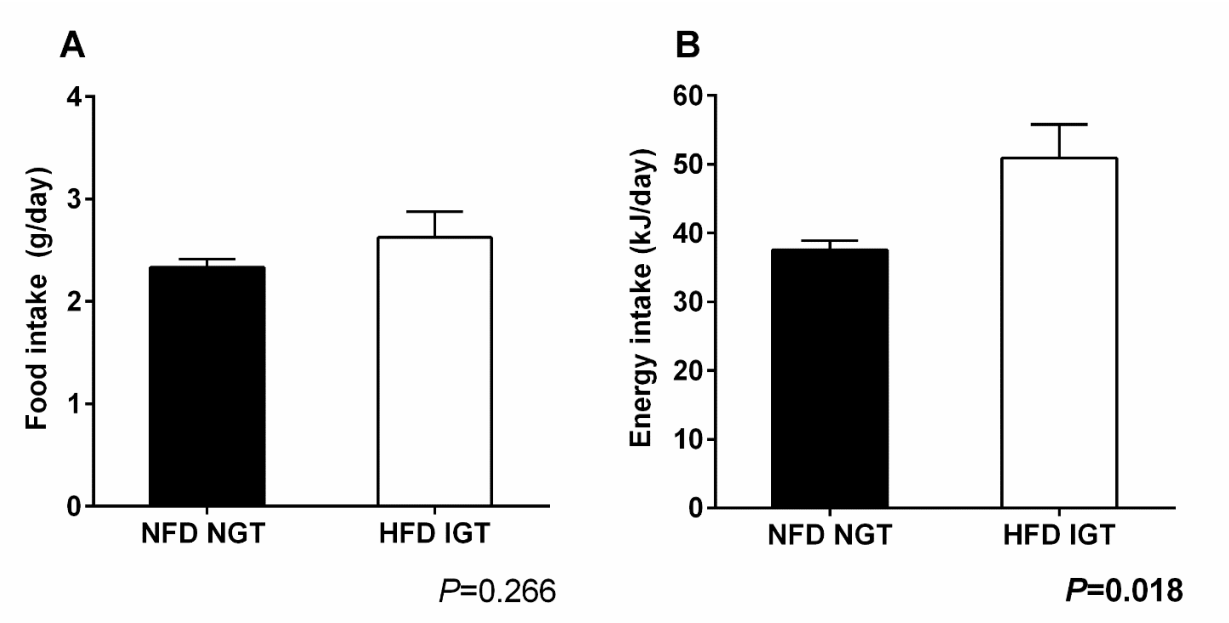


Figure 2.12 A Food intake and energy intake in pregnancy

A Daily food intake and **B** daily energy intake in NFD NGT dams and HFD IGT dams in pregnancy. Analysis by independent samples t-test. Values are mean ± SEM. *n*(NFD NGT)=6, *n*(HFD IGT)=6.

2.5.2.3 Glucose intolerance is not maintained in pregnancy

GTTs were performed in late gestation. There was no difference in glucose tolerance between the two dietary groups at either E15.5 (*P*=0.253; **Figure 2.13A**) or E18.5 (*P*=0.501; **Figure 2.13B**). Similarly, there was no difference in glucose AUC in late gestation (E15.5: NFD NGT 1663.1 ± 116.5 mmol/l.min, HFD IGT 1821.7 ± 74.6 mmol/l.min, *P*=0.267; E18.5: NFD NGT 1352.4 ± 76.2 mmol/l.min, HFD IGT 1413.5 ± 75.8 mmol/l.min, *P*=0.586).

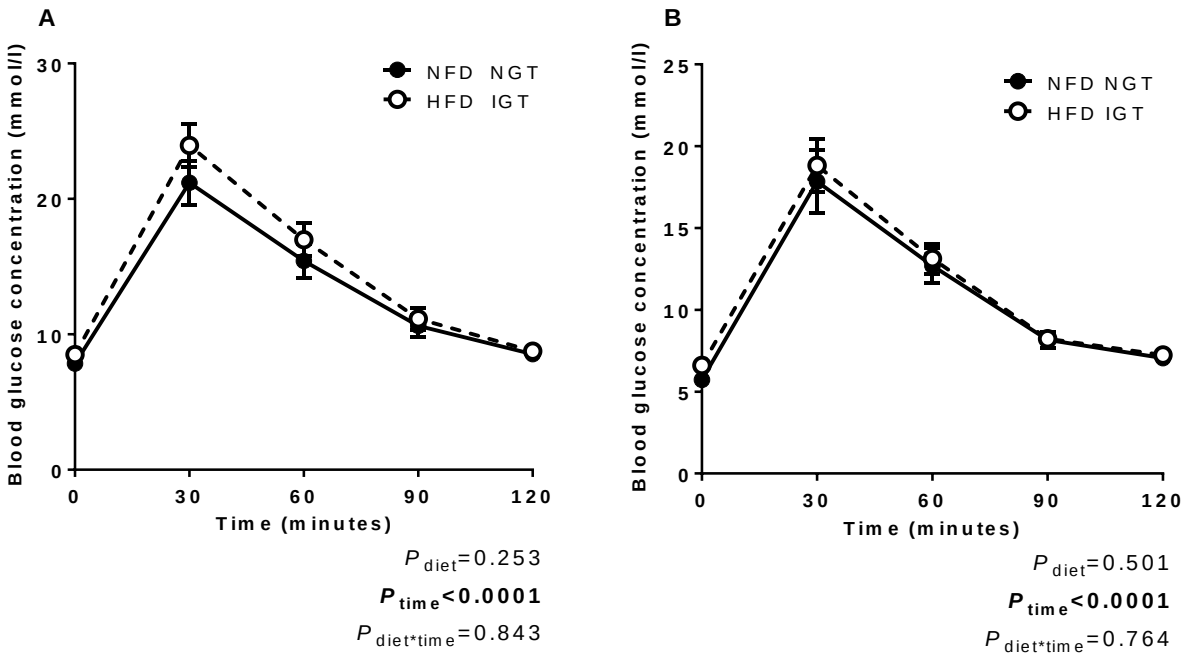


Figure 2.13 Glucose tolerance tests in late gestation
Mean GTT values in NFD NGT and HFD IGT dams at **A** E15.5 and **B** E18.5. GTT analysed by repeated measures ANOVA for maternal diet and time. Values are mean ± SEM. E15.5 *n*(NFD NGT)=9, *n*(HFD IGT)=10. E18.5 *n*(NFD NGT)=8, *n*(HFD IGT)=11.

To assess the effect of pregnancy status on glucose profiles, GTTs performed before pregnancy for both NFD NGT and HFD IGT dams were compared to those observed at E15.5 in the same animals. As expected, glucose intolerance was greater in HFD IGT mice (P_{diet}<0.0001), yet there was no observed effect of pregnancy status on glucose levels ($P_{gestation}$=0.191). As highlighted in **Figure 2.14**, glucose levels were found to increase from the non-pregnant to the pregnant state in NFD NGT mice, yet did not further increase in HFD IGT mice. Analysis of glucose AUC values supports this: glucose AUC levels increased by 24% in NFD NGT mice from the non-pregnant to the pregnant state yet remained unchanged in HFD IGT mice (NFD NGT pre-pregnancy 1337.0 ± 41.7 mmol/l.min, NFD NGT E15.5 1663.1 ± 97.9 mmol/l.min; HFD IGT pre-pregnancy 1837.4 ± 41.7 mmol/l.min,HFD IGT E15.5 1821.7 ± 97.9 mmol/l.min; $P_{gravida}$=0.078; P_{diet}<0.0001; **Figure 2.15**).

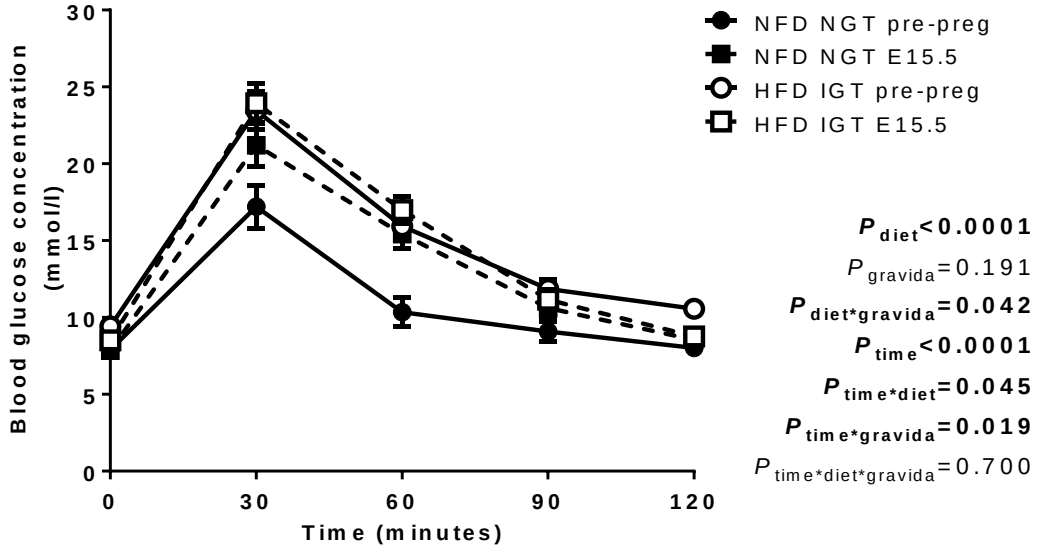


Figure 2.14 Comparing glucose tolerance tests prior to pregnancy and at E15.5
GTT values in NFD NGT mice prior to pregnancy and at E15.5, and in HFD IGT mice prior to pregnancy and at E15.5. GTTs analysed by repeated measures ANOVA for diet, gravida and time. Values are mean ± SEM. *n*(NFD NGT)=9, *n*(HFD IGT)=10.

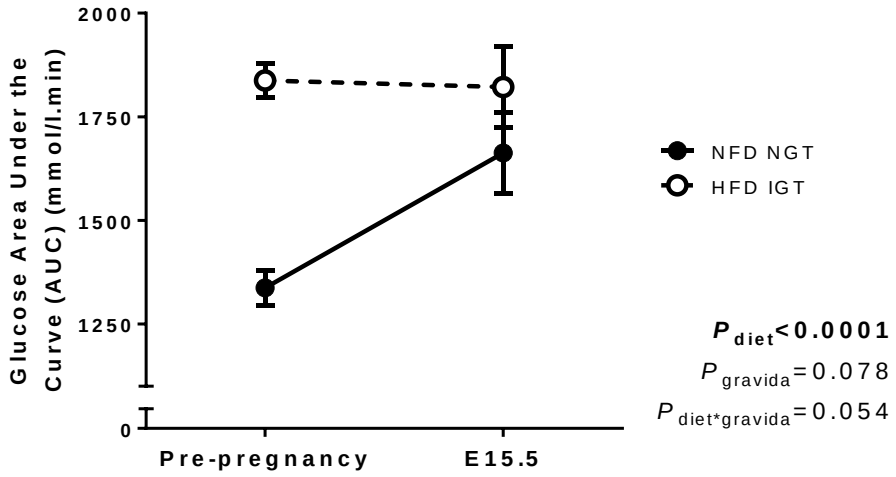


Figure 2.15 Comparing glucose AUC values prior to pregnancy and at E15.5
Glucose AUC in NFD NGT mice and HFD IGT mice prior to pregnancy and at E15.5. Analysis by repeated measures ANOVA for diet and gravida. Values are mean ± SEM. *n*(NFD NGT)=10, *n*(HFD IGT)=10.

GTTs were also performed in a cohort of mice at PN21 (weaning). There was no difference in glucose handling at weaning, assessed as either glucose tolerance curves (P_{diet}=0.682; **Figure 2.16**) or glucose AUC (NFD NGT 1257.6 ± 115.2 mmol/l.min, HFD IGT 1178.4 ± 145.7 mmol/l.min; *P*=0.432).

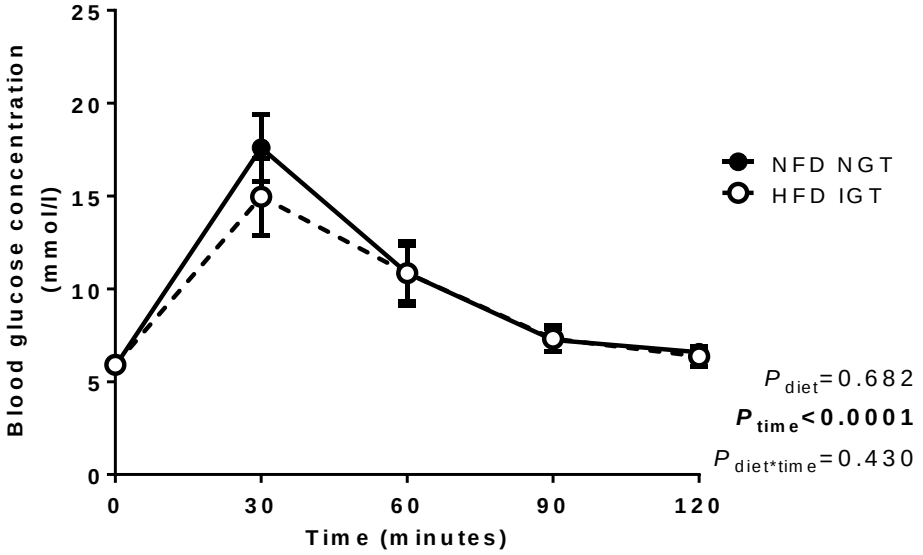


Figure 2.16 Glucose tolerance tests at PN21
GTT in NFD NGT and HFD IGT mice at PN21. Values are mean ± SEM. *n*(NFD NGT)=12, *n*(HFD IGT)=9.

2.5.2.4 High fat fed dams are hyperinsulinaemic

HFD IGT dams had 78% higher plasma insulin levels at E18.5 than NFD NGT dams (NFD NGT 0.36 ± 0.04 ng/ml, HFD IGT 0.64 ± 0.10 ng/ml; *P*=0.031, **Figure 2.17A**). Plasma leptin levels were similar in the two groups (NFD NGT 5.86 ± 0.74 ng/ml, HFD IGT 6.42 ± 1.51 ng/ml; *P*=0.747, **Figure 2.17B**).

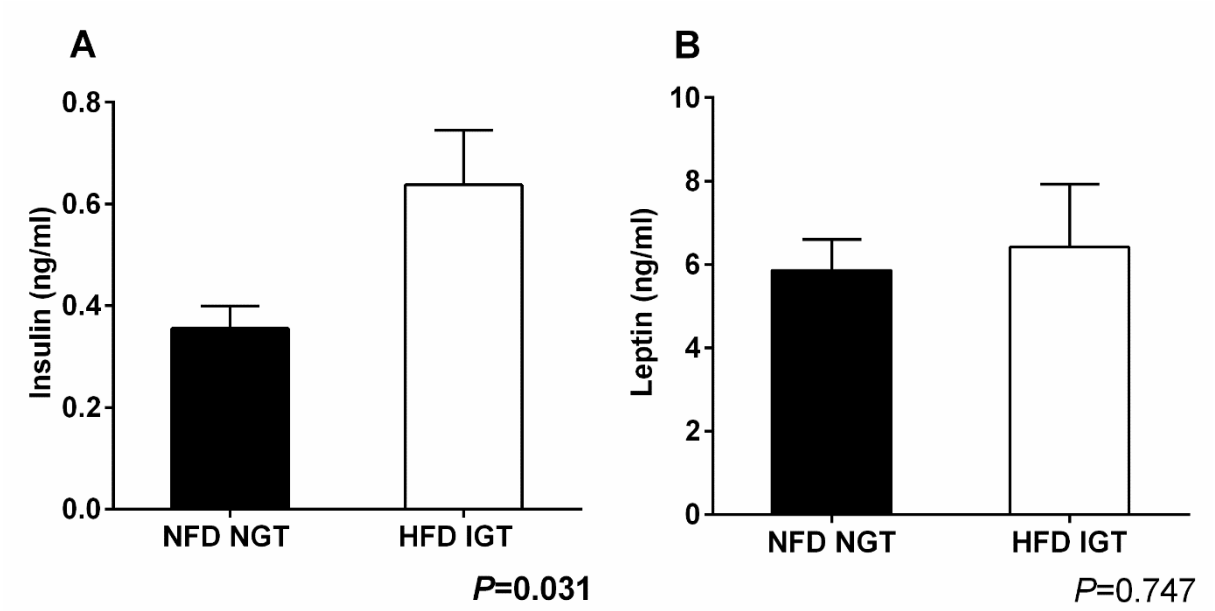


Figure 2.17 Maternal plasma insulin and leptin levels at E18.5
A Maternal plasma insulin and **B** plasma leptin at E18.5 in NFD NGT and HFD IGT dams. Analysis by independent samples t-test. Values are mean ± SEM. *n*(NFD NGT)=7, *n*(HFD IGT)=7.

2.5.2.5 Correlation analyses of maternal parameters

Associations between maternal parameters prior to pregnancy, at E0.5 and at E18.5 are presented in **Table 2.4**. Maternal glucose AUC prior to pregnancy had a positive correlation with maternal weight prior to pregnancy, maternal weight at conception (E0.5) and maternal insulin at E18.5. Maternal weight prior to pregnancy was associated with maternal weight at E0.5 and E18.5, and maternal weight gain was positively associated with maternal weight at E18.5. There were no other associations between maternal parameters.

Table 2.4 Spearman's rank coefficients for associations between maternal parameters prior to pregnancy, at E0.5 and at E18.5

		Weight pre-pregnancy	Weight E0.5	Weight E18.5	Weight gain	Glucose AUC pre-preg.	Glucose AUC E18.5	Insulin E18.5
Weight E0.5	**rho**	**0.872**						
	P	**<0.0001**						
Weight E18.5	**rho**	**0.749**	**0.731**					
	P	**<0.0001**	**<0.0001**					
Weight gain	**rho**	-0.032	-0.105	**0.460**				
	P	0.865	0.582	**0.009**				
Glucose AUC pre-pregnancy	**rho**	**0.550**	**0.349**	0.014	-0.220			
	P	**<0.0001**	**0.002**	0.939	0.234			
Glucose AUC E18.5	**rho**	0.103	0.109	-0.224	-0.403	0.015		
	P	0.665	0.657	0.485	0.190	0.950		
Insulin E18.5	**rho**	0.168	0.033	0.014	-0.317	**0.560**	-0.014	
	P	0.581	0.920	0.974	0.305	**0.033**	0.974	
Leptin E18.5	**rho**	0.108	0.134	-0.143	-0.387	0.088	0.214	0.456
	P	0.712	0.648	0.643	0.186	0.764	0.482	0.120

Spearman's rho (top value), *P* value (bottom value). Values in bold are statistically significant.

2.6 Discussion

Previous studies investigating the effect of maternal diabetes on offspring kidney development are limited to protocols that utilise STZ and which model uncontrolled Type 1 diabetes. These studies are typically associated with severe and persistent maternal fasting hyperglycaemia and profound offspring growth restriction and do not represent the metabolic conditions observed in women with pre-gestational Type 2 diabetes or gestational diabetes.

In the present study, female mice were fed a 21% high fat diet (or 6% fat matched diet) for 6 weeks to model pre-gestational Type 2 diabetes. GTTs performed before mating revealed variability in the degree of glucose intolerance in high fat fed mice – only half of the mice on the high fat diet exhibited glucose AUC values above that of normal fat fed mice. Based on these pre-pregnancy glucose AUC values, high fat fed mice with impaired glucose tolerance and normal fat fed mice with normal glucose tolerance were selected for inclusion in the study.

Previously, phenotypic variation in susceptibility to diet-induced diabetes and obesity has been recognised across different rodent strains (Surwit et al., 1988, Clee and Attie, 2007, Montgomery et al., 2013) however studies of diabetes-prone and diabetes-resistant mice within the one strain or colony are lacking. Studies of diet-induced maternal diabetes have, to our knowledge, made no reference to selecting or excluding dams based on metabolic phenotype. In the present study body weight and weight gain did not predict glucose intolerance in high fat fed mice. Diabetes-susceptible HFD IGT mice may have differed in liver lipid accumulation and insulin signalling compared with HFD NGT mice (Montgomery et al., 2013) although these parameters were not investigated. Glucose intolerance was not observed after 3 weeks of high fat feeding but was present after 5 weeks. Extending the duration of high fat feeding beyond 5 weeks may have helped improve the rate of glucose intolerance in high fat fed mice, but was avoided to minimise the effects of long-term fat feeding, extreme levels of obesity and reduced reproductive success (Bermejo-Alvarez et al., 2012).

During the 6 week dietary protocol, selected HFD IGT mice displayed greater energy intake and weight gain then NFD NGT mice. Just prior to mating, HFD IGT mice were heavier than NFD NGT mice and had increased adiposity. Excess maternal weight was associated with mild fasting hyperglycaemia and substantial glucose intolerance. Previous protocols of high fat feeding in rodents vary from 4 to 14 weeks and have been associated with an 11-81% increase in body weight prior to pregnancy (Holemans et al., 2004, Srinivasan et al., 2006b, Ferezou-Viala et al., 2007, Bruce et al., 2009, Nivoit et al., 2009, Li et al., 2013). Unfortunately, in most of these studies pre-pregnancy glucose tolerance was rarely stated and only a few publications report elevated fasting glucose ranging from 7% to over 50% in high fat mice before mating (Holemans et al., 2004, Nivoit et al., 2009, Li et al., 2013).

In pregnancy, HFD IGT dams maintained an increased energy intake. However, body weight did not differ between the two dietary groups at E18.5 with data suggesting that this was most likely related to HFD IGT dams gaining less weight in late gestation, a feature often observed in obese women in pregnancy (Siega-Riz and Gray, 2013). This is supported by rodent studies from Liang et al. (2009), Nivoit et al. (2009), and Samuelsson et al. (2013) but contradicts a number of papers which report increased body weight in late gestation (Holemans et al., 2004, Srinivasan et al., 2006b, Jones et al., 2009, Ashino et al., 2012, Li et al., 2013). Previous reports of glucose levels in pregnant high fat or 'Western' diet fed animals are also ambiguous, with studies variously citing an increase in fasting glucose and glucose intolerance (Srinivasan et al., 2006, Holemans et al., 2004) or no difference (Liang et al., 2009, Jones et al., 2009) compared with controls.

Unexpectedly, the marked discrepancy in glucose tolerance between the two groups before mating was not evident at E15.5 despite HFD IGT dams maintaining a greater body weight. Glucose AUC levels increased in NFD NGT mice between the non-pregnant state and E15.5 to match that of HFD IGT mice, highlighting that pregnancy itself is a state of glucose intolerance. Glucose levels were unexpectedly stable in HFD IGT mice between pre-conception and E15.5, indicating that HFD IGT dams had reached an upper limit or threshold of glucose intolerance after 6 weeks of high fat feeding that was not exacerbated by pregnancy. GTTs were only performed on the day of embryo collection (either E15.5 or E18.5) to limit excessive stress on the dams. Consequently, the duration of glucose intolerance in HFD IGT dams and the developmental stage at which offspring were no longer exposed to glucose levels significantly greater than that of controls is unfortunately unknown. Glucose tolerance curves decreased uniformly in both groups between E15.5 and E18.5, possibly due to dams fasting in preparation for birth. Despite normal glucose tolerance and body weight at E18.5, HFD IGT dams were hyperinsulinaemic in late gestation as previously reported in rodent studies of high fat feeding (Holemans et al., 2004, Jones et al., 2009, Liang et al., 2009a, Ashino et al., 2012, Samuelsson et al., 2013).

While the dietary protocol was continued throughout lactation, body weight and glucose tolerance were surprisingly similar in NFD NGT and HFD IGT dams at PN21. Previous studies utilising high fat and 'Western' diets do not commonly report body weight or glucose and insulin levels in lactating dams, and those that do reveal equivocal effects (Ferezou-Viala et al., 2007, Samuelsson et al., 2008, Nivoit et al., 2009, Cerf and Louw, 2010, Ashino et al., 2012, Jackson et al., 2012). In the present study, NFD NGT dams showed comparable glucose AUC before mating and at PN21, yet there was a marked drop in glucose AUC values in HFD IGT dams after birth compared to pre-conception. Dams were housed with their pups and were still lactating during the GTT at PN21 which may have confounded the glucose curves obtained.

The metabolic phenotype observed in the present study has both similarities and discrepancies to previous publications. As discussed, the differences are numerous and may relate to the age of mice when placed on diet, the length of time on diet before mating, general animal housing conditions, the strain or species studied and the method employed to measure glucose levels. The composition of both control and high fat diets (whether fat was derived from animal or vegetable products; the calculated digestible energy from lipid, carbohydrate and protein; vitamin and mineral composition; specific fatty acid composition; and the addition of simple sugars) are also important considerations and add to the complexity when comparing this study to previous studies of pre-gestational Type 2 diabetes.

To summarise, the findings of the studies described in the present chapter characterise a mouse model of diet-induced obesity-associated pre-gestational Type 2 diabetes. Mice fed a 'Western' style high fat diet for 6 weeks were overweight with mild fasting hyperglycaemia and considerable glucose intolerance prior to pregnancy. Contrary to what was hypothesised, glucose intolerance was not maintained in late gestation despite HFD IGT dams being overweight at E15.5 and hyperinsulinaemic at E18.5. The duration of glucose intolerance in high fat fed dams, and therefore the length of gestation in which embryos were exposed to excess glucose, is unknown. This will be an important consideration when evaluating offspring kidney development which is well underway in late gestation. Kidney development and renal health in offspring of mice with pre-gestational obesity and glucose intolerance are investigated in the studies described in Chapter 3.

CHAPTER THREE

HIGH FAT FEEDING IN MICE AS A MODEL OF PRE-GESTATIONAL TYPE 2 DIABETES – EFFECTS ON OFFSPRING KIDNEY DEVELOPMENT AND RENAL FUNCTION

3.1 Introduction

An increasing number of pregnancies are complicated by overweight/obesity and associated maternal glucose intolerance. Maternal glucose intolerance poses both short and long-term complications for offspring including neonatal macrosomia, hypoglycaemia and birth trauma, as well an increased risk of diabetes, obesity and hypertension in adulthood (HAPO Study Cooperative Research Group, 2008, Simeoni and Barker, 2009, Hay, 2012). Similar adverse outcomes are observed in pregnancies complicated by maternal obesity in isolation (Jensen et al., 2003, Boney et al., 2005) and in combination with maternal glucose intolerance (Catalano et al., 2012, Langer, 2015). There is now a growing, albeit limited, body of evidence indicating that maternal diabetes and obesity may adversely affect offspring kidney development and adult renal function.

It is well recognised that infants of diabetic mothers, whether it be Type 1, Type 2 or gestational diabetes, have an increased risk of congenital abnormalities of the kidney and urinary tract (Martinez-Frias, 1994, Martínez-Frías et al., 1998, Sheffield et al., 2002, Kamdem et al., 2005, Nielsen et al., 2005, Bánhidy et al., 2010, Davis et al., 2010, Shnorhavorian et al., 2011). Recently, a study by Hsu et al. (2014) further identified an increased risk of CKD in children of women with pre-gestational diabetes, gestational diabetes and obesity. In addition to renal structural malformations, it was previously hypothesised that maternal diabetes may have more subtle effects including adversely altering kidney cell populations and reducing nephron endowment. As nephrogenesis is complete by approximately 34-36 weeks in humans, intrauterine perturbations such as hyperglycaemia will likely lead to permanent deficits in nephron endowment. In turn, a low nephron endowment is associated with an increased risk of glomerular hyperfiltration, glomerulosclerosis, systemic hypertension and renal failure; as proposed by the 'Brenner Hypothesis' (Brenner et al., 1988). Current techniques limit the quantification of nephron number in humans to autopsy tissue, and have prevented the assessment of nephron endowment in offspring exposed to maternal hyperglycaemia and glucose intolerance. However, reduced renal function and increased ESRD have been reported in offspring of Type 1 diabetic mothers (Abi Khalil et al., 2010) and in diabetic offspring of Type 2 diabetic mothers (Nelson et al., 1998b, Pavkov et al., 2010), suggestive of a reduced nephron endowment.

Experimental studies of maternal diabetes and its effects on offspring kidney development and renal function are mostly limited to models of Type 1 maternal diabetes. These studies utilise STZ, a glucosamine-nitrosurea compound that acts as an alkylating agent, which selectively targets pancreatic islet β cells, leading to insulin depletion and severe and persistent fasting hyperglycaemia. In rodents, offspring of STZ-induced diabetic dams display a deficit in nephron number proportionate to the magnitude of maternal hyperglycaemia (Amri et al., 1999, Tran et al., 2008, Hokke et al., 2013).

Unlike the human condition, offspring of STZ-treated dams are commonly growth restricted, which further exacerbates and confounds interpretation of the deficit in nephron endowment. Previous studies have identified a positive association between offspring birth weight and nephron number (Hokke et al., 2013), however kidney development in macrosomic offspring exposed to maternal glucose intolerance has yet to be investigated. Long-term studies in adult offspring of maternal diabetes are also limited to the STZ paradigm. Rodent offspring are reported to exhibit proteinuria and reduced renal function, as well as glomerular hypertrophy and hypertension (Rocha et al., 2005, Magaton, 2007, Nehiri et al., 2008, Chen et al., 2010, F. et al., 2013, Yan et al., 2014). Offspring also display perturbed glucose handling (Magaton, 2007, Chen et al., 2010) and greater body weight (Rocha et al., 2005, Nehiri et al., 2008). These observations in rodents recapitulate the limited studies in humans.

Experimental studies of STZ-induced maternal diabetes provide evidence of developmental programming of the kidney, yet only model the condition seen in a minority of women with uncontrolled Type 1 diabetes complicated with pre-eclampsia and/or vascular disease. Studies assessing kidney development and renal function in offspring exposed to the more commonly encountered conditions of pre-gestational Type 2 diabetes and gestational diabetes are lacking, but are urgently needed given the rising prevalence of obesity and maternal glucose intolerance which is often present in women before conception.

The maternal phenotype in a mouse model of diet-induced pre-gestational Type 2 diabetes was characterised in Chapter 2. High fat fed mice were found to have increased body weight and exhibited glucose intolerance prior to conception. Glucose tolerance was found to be similar to normal fat fed dams in late gestation and unfortunately it is unknown when in gestation glucose tolerance became equivalent in high fat and normal fat fed dams. The studies described in the present chapter will investigate the immediate and long-term effects of obesity-associated pre-gestational Type 2 diabetes on offspring growth, kidney development and adult metabolic and renal health. Offspring of high fat fed dams were assessed during the embryonic and early postnatal periods for evidence of altered growth and kidney development. Ureteric branching morphogenesis and nephrogenesis were assessed as they are key processes in kidney development. Parameters of metabolic health, including obesity, glucose intolerance and body composition, were assessed in offspring throughout adulthood. Renal function and morphology were also measured in adult offspring in order to examine the long-term consequences of exposure to obesity-associated pre-gestational Type 2 diabetes.

3.2 Aims

- To assess if exposure to obesity-associated pre-gestational Type 2 diabetes affects growth and kidney development in offspring during embryogenesis and in early postnatal life.
- To assess if exposure to obesity-associated pre-gestational Type 2 diabetes affects body growth, glucose tolerance and body composition in adult offspring.
- To assess if perturbations in kidney development have long-term consequences on renal function and morphology in adult offspring.

3.3 Hypotheses

- Compared with offspring of normal fat fed dams, offspring of dams with diet-induced overweight and glucose intolerance will be macrosomic and have deficits in kidney development relative to body weight.
- Adult offspring of dams with pre-gestational glucose intolerance will be overweight and demonstrate increased fat composition and impaired glucose tolerance.
- Adult offspring will exhibit reduced renal function and altered renal morphology.

3.4 Methods

3.4.1 Tissue collection

Offspring of dams that met the glucose AUC criteria (see **Section 2.5.1.4**) were collected at three time points for analysis of kidney development; E15.5, E18.5 and PN21. Kidneys were collected at E15.5 for analysis of ureteric branching by Optical Projection Tomography (OPT). E15.5 represents a period of early nephron induction and mid-late ureteric tree development where the kidney is suitable for OPT analyses because the kidney capsule is relatively thin allowing for antibody penetration, and the increasingly dense peripheral ureteric tips can still be spatially resolved using OPT image deconvolution software (Short et al. 2010). Kidneys were collected at E18.5 for estimation of nephron number. This is the latest time point to assess nephrogenesis before birth (E19.5), allowing assessment of the maximal effect of the maternal environment on prenatal nephrogenesis. At PN21 final nephron endowment was determined (nephrogenesis is complete by approximately PN5 in the mouse). At this time distinctive zones of the medulla and cortex can be observed and is the end of the lactation period.

3.4.1.1 E15.5 and 18.5 tissue collection

Pregnant dams at E15.5 and E18.5 were anaesthetised using Isoflurane in an induction chamber. When deeply anaesthetised (absence of pedal reflex) an incision was made along the thoracic wall and through the diaphragm and ribcage to expose the heart. Mice were exsanguinated by left ventricular puncture using a 26-gauge needle with syringe. Approximately 500-1000 µl of blood was collected into an Eppendorf tube containing EDTA to prevent coagulation and centrifuged for 10 minutes at 4°C at 3000rpm. Maternal plasma was aspirated from packed cells and both were frozen at -80°C.

The incision was next extended along the anterior abdominal wall to expose the uterine horns, which were carefully excised and placed into a dish containing phosphate-buffered saline (PBS, pH 7.4). The uterine horns were examined for embryo number and evidence of embryo resorption. At E15.5 amniotic fluid from the amniotic sac was aspirated and frozen at -80°C. Insufficient volumes of amniotic fluid surrounding each embryo prevented collection of amniotic fluid at E18.5. Individual embryos were carefully removed from the uterus and surrounding amniotic membrane. Placentae were weighed and alternate placentae were frozen and stored at -80°C or fixed in 1 ml 4% paraformaldehyde (w/v) in PBS (PFA, Sigma Aldrich, Castle Hill, NSW, Australia). Fetuses were weighed and digital callipers (Mitutoyo Digimatic 8” Calipers, Tokyo, Japan) used to measure crown-rump length and head diameter. Fetuses were decapitated prior to collection of 10-50 µl of fetal blood (E18.5 embryos only). Fetal blood was centrifuged for 10 minutes at 4°C at 3000 rpm, and fetal plasma was aspirated from packed cells and both were frozen at -80°C.

Embryos were placed on a silicon-coated petri dish filled with PBS. With the aid of a dissecting microscope (SZX12 Stereo Microscope, Olympus, Australia), the abdominal wall was opened. Embryos were sexed based on the appearance of the gonads. Kidneys were carefully removed and fixed in 1 ml PFA. E15.5 kidneys were fixed for 20 minutes, transferred to PBS and stored at 4°C. E18.5 kidneys were fixed for 6 hours and transferred to 70% ethanol.

3.4.1.2 PN21 tissue collection

Dams that formed the PN21 cohort were allowed to litter down naturally. Due to the high prevalence of cannibalism in both normal and high fat fed dams (45%), pups were not weighed or sexed until PN3 to minimise disruption to the nest. Offspring were weighed approximately every second or third day thereafter. At PN21, pups were weighed and one to two pups of each sex, with body weights closest to the litter mean, were separated from the litter. Body composition was measured by DEXA scans in PN21 mice as previously described (**Section 2.4.5**).

PN21 offspring were anaesthetised in an induction chamber and anaesthesia maintained using Isoflurane. Following absence of pedal reflex, an incision was made along the thoracic wall and through the diaphragm and ribcage to expose the heart. Approximately 200 µl of blood was collected from the right atrium using a 26-gauge needle and syringe and collected into an Eppendorf tube containing EDTA. Blood was centrifuged for 10 minutes at 4°C at 3000 rpm and plasma then aspirated and frozen at -80°C. The incision was extended along the abdominal wall and through the peritoneum. The abdominal aorta and kidneys were carefully exposed with cotton tips. Using a winged infusion set, a 25-gauge butterfly needle connected to a 50 ml syringe filled with PBS was inserted into the left ventricle of the heart. The inferior vena cava proximal to the kidneys was then cut using fine spring scissors and 20 ml of PBS at 5 ml/min was administered through the left ventricle to clear the circulation and kidneys of blood. Once cleared, a 50 ml syringe filled with paraformaldehyde-glutaraldehyde solution (Karnovsky's Fixative) was connected to the infusion set and 20 ml was administered at 5 ml/min. Following fixation, left and right kidneys were carefully excised, weighed and placed in formalin.

3.4.2 Plasma analyses

Insulin and leptin protein content were quantified in E18.5 fetal plasma samples by ELISA (see **Section 2.4.8)**. Due to insufficient plasma volumes from individual fetuses, plasma samples were pooled. Equal volumes of plasma from 2-3 fetuses from within the one litter and of the same sex were pooled. Data are presented per litter, where each pooled sample represents *n*=1.

3.4.3 Assessment of ureteric tree development

Whole E15.5 kidneys were immunostained for the ureteric epithelium and scanned by OPT. 3D representations of the ureteric tree were analysed by software for quantification of branch length and ureteric tip number. Specific methodologies are outlined in detail below and are adapted from protocols described previously (Short and Smyth, 2012, Hokke et al., 2013, Short et al., 2013).

3.4.3.1 Wholemount staining protocol

Kidneys in 1.5 ml Eppendorf tubes were transferred from PBS to Tris-buffered saline (TBS; Sigma-Aldrich, Castle Hill, NSW, Australia). Kidneys were placed overnight in 200 µl TBS-Triton X100 (TBS-Tx; Sigma-Aldrich, Castle Hill, NSW, Australia) + 1% Bovine Serum Albumin (w/v) (BSA; Sigma-Aldrich, Castle Hill, NSW, Australia) + 10% donkey serum (Sigma-Aldrich, Castle Hill, NSW, Australia) to prevent non-specific binding. Kidneys were then incubated for 48 hours with TROP-2 primary antibody (Mouse TROP-2 polyclonal Goat IgG; R&D systems, Minneapolis, MN, USA) at a concentration of 10 µg/ml in 200 µl of blocking solution (TBS-Tx +1% BSA +10% donkey serum). TROP-2 is a cell surface glycoprotein expressed in branching ureteric epithelium (Tsukahara et al., 2011, Combes et al., 2014). Kidneys were subsequently washed three times in 1 ml TBS-Tx for 30 minutes before being carefully transferred to 15 ml falcon tubes and washed in 10 ml TBS-Tx overnight to remove excess primary antibody. After being transferred back to 1 ml Eppendorf tubes, kidneys were incubated overnight with Alexa Fluor 647 Donkey anti-Goat IgG secondary antibody (Molecular Probes, Life Technologies, Mulgrave, VIC, Australia) at a 1:400 dilution in 200 µl TBS-Tx + 1% BSA. Kidneys were washed three times in 1 ml volumes of TBS-Tx for 30 minutes and once in 10 ml TBS-Tx overnight to remove excess secondary antibody, and then transferred to TBS. All incubations were performed at 4°C on a rocking platform mixer. All solutions used in this protocol were filtered to eliminate particles and lint that auto-fluoresce and interfere with scanning.

3.4.3.2 Tissue embedding and mounting

Stained E15.5 kidneys were examined under a dissection microscope and lint and foreign bodies were carefully removed using fine forceps. Kidneys were subsequently embedded in 1% agarose in distilled water (low melting point, analytical grade agarose; Promega, Alexandria, NSW, Australia) (**Figure 3.1**). Specifically, agarose was filtered using a 0.45 µm cartridge filter into a 6-well culture plate. The agarose was cooled to 30°C and individual kidneys were carefully pipetted into a well of agarose. Using a rounded glass hook, surrounding agarose was gently swirled to equilibrate the kidney with the mounting medium, and kidneys were positioned in the centre of the well. When set, agarose rounds were cut out of the wells and trimmed using a scalpel blade to form rectangular blocks with a base approximately 10 mm square and sides 15 mm in length, with the embedded kidney 5 mm from the

top and 10 mm from the base (Short and Smyth, 2012). The rectangular block was then trimmed using a scalpel blade to form a cone shape to minimise the volume of agarose surrounding the kidney.

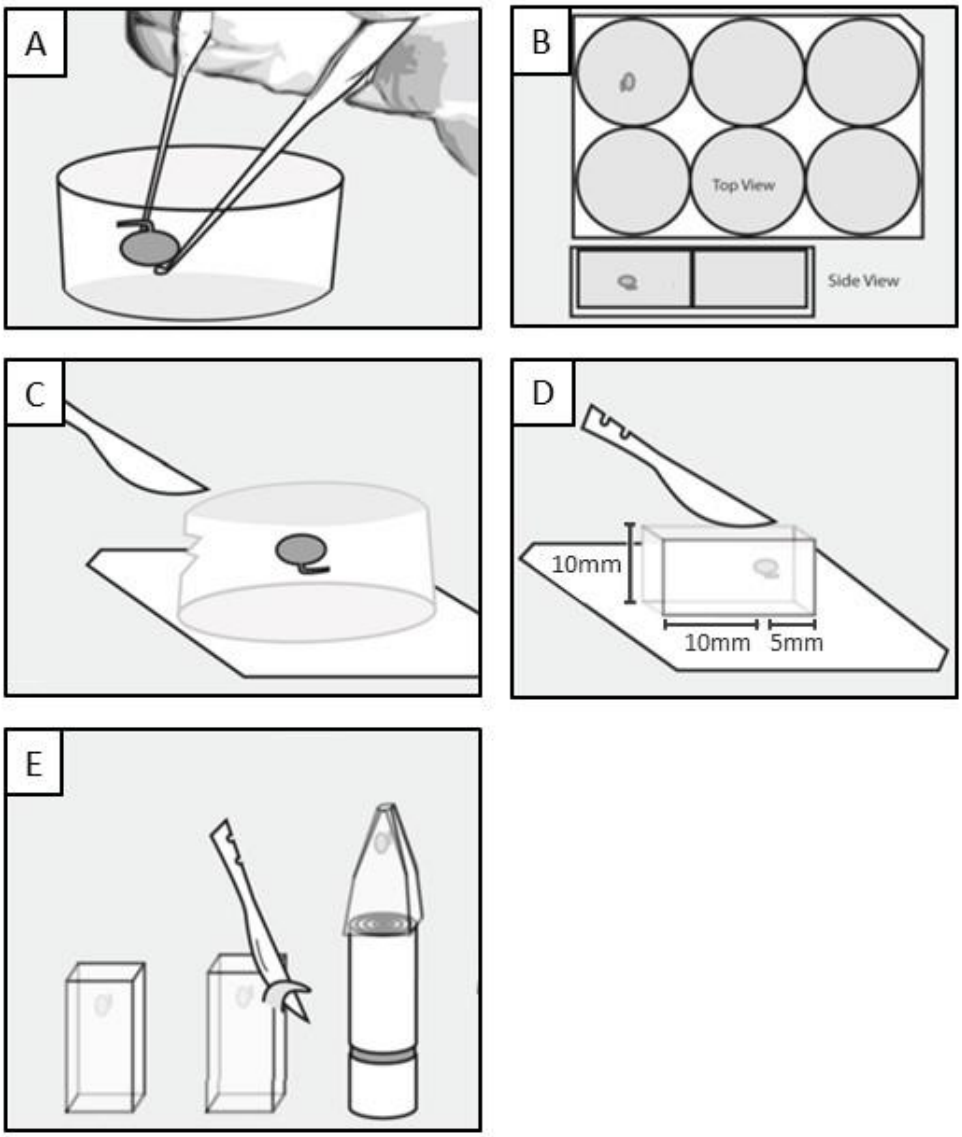


Figure 3.1 OPT embedding.
Diagram illustrating the embedding of a kidney for OPT imaging. **A** A kidney is placed in a well of molten agarose and **B** positioned with glass hooks in the middle of the well. **C** Once set, agarose rounds were cut out of the well and **D** the surrounding agarose was trimmed with a scalpel blade to form a rectangular block, with the kidney centred at one end. **E** The block is trimmed to form a cone shape that will be later affixed to a metal OPT mount. Figure adapted from Short and Smyth (2012).

Trimmed agarose blocks were placed in 50 ml falcon tubes and dehydrated in methanol for 2x12 hours. Kidneys were then optically cleared for 2x12 hours in benzyl alcohol/benzyl benzoate (BABB; Sigma-Aldrich, Castle Hill, NSW, Australia) (1:2 mixture). BABB is a clearing agent that allows agarose to

become transparent and minimises scattering through the specimen as its refractive index is similar to tissue. Once cleared, agarose blocks were glued onto metal OPT mounts.

3.4.3.3 Scanning and reconstruction

Kidneys were imaged in a Skyscan Bioptonics 3001 OPT scanner (Bioptonics, Edinburgh, UK). On each day of imaging, the scanner was calibrated with a metal alignment pin and calibration measurements were performed using Skyscan software (Skyscan, Kontich, Belgium). Mounted kidneys were inverted, placed onto a magnetic stage in the scanner and lowered into a cuvette containing BABB. The mount was positioned to identify the centre of rotation of the kidney using Skyscan software. Exposure times were adjusted.

Two scans were undertaken per specimen – one at 9° intervals to acquire 40 images through a 360° axial rotation (serving as a reference scan for *x-y* alignment), and one at 0.45^{o} intervals to acquire 800 images through a 360° axial rotation (used to assess ureteric tree development). Kidneys were scanned using a 647 nm fluorescent channel. To generate each image (**Figure 3.2A**), emitted light was detected with a back-illuminated CCD camera with a resolution of 1024 x 1024 pixels.

Angular 2D projections of the ureteric tree were computationally reconstructed using N-Recon software (Skyscan, Kontich, Belgium). An *x-y* alignment was performed with the reference scan to correct for any shifts in the main scan. Using a filtered back-projection algorithm, the corrected and normalised images were then reconstructed with unified misalignment compensation to create horizontal cross-sectional slices through the kidney (*z*-series) thus generating 3D voxel (volume pixel) information of the native ureteric epithelial tree. Reconstructed 3D datasets were visualised and rendered using Drishti software (version 2.4, Australian National University, ANUSF VizLab, Canberra, ACT, Australia) (**Figure 3.2B**).

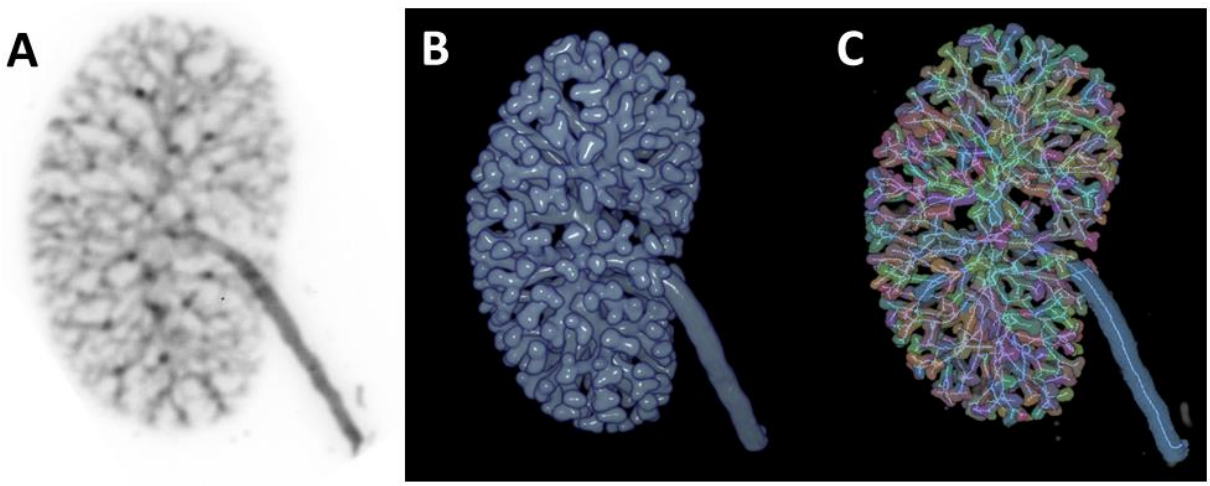


Figure 3.2 Optical Projection Tomography
A OPT generated 2D image of a whole E15.5 kidney stained fluorescently for TROP-2, a marker of ureteric epithelium. **B** Reconstructed OPT dataset: 3D data set of a reconstructed ureteric epithelium visualised and rendered with Drishti software. **C** Analysis of OPT dataset with Tree Surveyor software to quantify ureteric tree development.

3.4.3.4 Quantitative assessment of ureteric tree development

Quantitative assessment of the ureteric epithelium was performed using Tree Surveyor software (version 1.0.8.20, Short et al. (2013)) (**Figure 3.2C**). Tree Surveyor software refines the complex scanned volume into a skeleton, from which measures of ureteric branch points, ureteric tip number, tree length and tree volume can be quantified. To do this, the software performs a watershed fill in *x-y-z* dimensions to produce a filled greyscale volume of the scanned dataset. This is then Gaussian blurred in 3D to establish vectors that are analysed to define a convergent, weighted centre of the branching volume and thus define a skeletal dataset (Short et al., 2013). The fully connected object skeleton is traversed from the user-defined root point of the tree (the ureter), to identify segments, segment lengths, branch points and ureteric tips (terminal end points) and a star-convex fill is applied to quantify segment and total tubular volumes that are exported in a summary spreadsheet.

3.4.4 Assessment of nephron number at E15.5

When counting structures such as glomeruli in histological sections it is essential that these structures can be unambiguously recognised regardless of the plane of sectioning. In adult kidneys, glomeruli are easily recognised by their characteristic capillary tuft, urinary space, and surrounding Bowman's capsule. However, in developing kidneys, glomeruli are at different stages of development with complex shapes and varying sizes making identification more difficult. In the present study, E15.5 kidneys were serially sectioned and then histochemically stained using a lectin (Peanut Agglutinin, PNA) which specifically binds to galactosyl-β (1,3)-N-acetyl galactosamine, a sugar moiety of cellular

and extracellular glycopeptides. These glycoproteins are expressed by glomerular podocytes as they mature, allowing the unambiguous identification of glomeruli from the S-shaped body stage of nephron development to the fully mature glomerulus (Cullen et al., 2000, Cullen-McEwen et al., 2012). All sections were then viewed as described below and all glomeruli counted.

3.4.4.1 Tissue processing and sectioning

Absolute nephron number was determined at E15.5 using whole-mount kidneys that were previously scanned by OPT. The agarose embedded kidneys were trimmed from metal OPT mounts and washed for 2x12 hours in methanol in 50 ml Falcon tubes to remove residual BABB. Agarose blocks were transferred to PBS overnight and then immersed in 30% sucrose in PBS overnight. Falcon tubes were subsequently incubated in a water bath to 37%. The agarose was carefully removed from the kidney and the kidneys rinsed in PBS and then transferred to 70% ethanol. The kidneys were then processed to paraffin (Leica PELORIS II TissueProcessor, Leica Microsystems, North Ryde, NSW, Australia) using a standard 1 hour processing cycle.

Paraffin blocks were serially sectioned (MicroTec CUT 4060 Rotary Microtome, Walldorf, Germany) at 4 µm, and all sections were mounted on Polysine coated slides (Menzel-Glaser, Braunschweig, Germany). Approximately 100-150 sections were collected per E15.5 kidney.

3.4.4.2 Histochemistry

Slides underwent histochemical staining for PNA. Sections were deparaffinised in xylene for 6 minutes, rehydrated in ethanol for 6 minutes, briefly transferred to water and washed in PBS. Sections were then incubated in 2% H_2O_2 in methanol for 10 minutes to neutralise any endogenous peroxidases. After a 5 minute wash in PBS, a 100 µl solution of neuraminidase type III from *Vibrio cholera* (Sigma-Aldrich, Castle Hill, NSW, Australia), [0.1 units/ml in 1% $CaCl_2$ in PBS] was added to each slide. Slides were cover-slipped and incubated for 30 minutes at 37°C in a humidibox. Neuraminidase cleaves N-acetylneuraminic acid from the carboxylic terminal of the sugar component of glycoproteins, increasing the availability of the receptor sequence for PNA binding. This and all subsequent incubations were performed using 100 µl solutions under a glass coverslip.

Following neuraminidase digestion, sections were washed in PBS for 10 minutes and incubated in 2% BSA in 0.3% triton in PBS for 30 minutes at room temperature. Triton acts as a detergent to permeabilise cell membranes, while BSA blocks non-specific binding. After blocking, biotinylated PNA lectin derived from *Arachis hypogaea* (Sigma-Aldrich, CastleHill, NSW, Australia) [20 mg/ml in 0.3% triton in PBS with 0.1 M combined ions ($MgCl_2$, $MnCl_2$, $CaCl_2$)] was added and slides incubated overnight at 4°C in a Perspex humidibox.

Following incubation with PNA, slides were rinsed in PBS for 10 minutes. Biotinylated PNA was visualised using the Elite streptavidin/biotin amplification ABC kit (Vector Laboratories Inc., Burlingame, CA, USA). 100 µl of ABC solution (1 µl of reagent A and 1 µl of reagent B/ 100 µl of 0.3% Triton in PBS) was added to each slide, and slides were incubated for 1 hour at room temperature. Slides were washed in PBS for 10 minutes and the stain was developed with 0.5 mg/ml diaminobenzidine (DAB) in distilled water (Dako, Botany, NSW, Australia) activated with 0.01% H_2O_2. Briefly, 100 µl of DAB was individually added to each slide and the development of the stain was monitored under a light microscope. As sections began to display localised brown staining of the glomerular podocytes (Figure 3.1) the reaction was ended by placing the slide in PBS.

Sections were counterstained with Harris's Haematoxylin for 30 seconds. Slides were rinsed in tap water for 30 seconds followed by Scott's tap water for 3 seconds before being rinsed again for 30 seconds in running tap water. Sections were dehydrated in ascending concentrations of ethanol over 6 minutes and xylene over 6 minutes, and coverslipped with dibutyl polystyrene xylene (DPX) (BDH Laboratory Suppliers, Poole, Dorset, UK).

3.4.4.3 Glomerular counting

To count glomeruli in an E15.5 kidney, the section with the largest cross-sectional area was projected onto A3 paper at a magnification of 150X (10X lens and 15X projection arm) in a darkened room using a light microscope (Olympus BX50) equipped with a projection arm. The perimeter of the section was traced and PNA-positive glomeruli were circled. The subsequent section was then projected on top of the trace and new PNA-positive glomeruli were identified and circled. This was continued in subsequent sections throughout the remaining tissue. Once the tissue was exhausted, the initial reference section was projected again. Moving in the opposite direction, new PNA-positive glomeruli were identified and circled in each preceding section. The total number of PNA-positive glomeruli were tallied per kidney.

3.4.5 Assessment of nephron number at E18.5 and PN21

Glomerular number in E18.5 embryos and PN21 offspring was determined using the physical disector/fractionator unbiased stereological method described by Cullen-McEwen et al. (2012). As with E15.5 kidneys, paraffin sections were histochemically stained with PNA to aid glomerular identification. However, kidneys at E18.5 and PN21 contain many more glomeruli than at E15.5, making the glomerular counting method used for E15.5 kidneys inefficient. Stereology was therefore used.

The physical disector/fractionator principle is considered the gold-standard method for estimating total glomerular number (Nyengaard, 1999, Bertram, 2001). This method is considered unbiased as it requires no knowledge or assumption of glomerular geometry such as size, shape or size distribution.

Using the disector principle (Sterio, 1984), glomeruli are sampled (counted) in a known fraction of kidney tissue, thereby increasing efficiency. Moreover, the fractionator design overcomes a number of technical artefacts that confound other stereological counting methods such as section compression and tissue shrinking/swelling caused by tissue processing.

3.4.5.1 Tissue processing and sectioning

Fixed kidneys were transferred to 70% ethanol and processed to paraffin (Leica PELORIS II TissueProcessor, Leica Microsystems, North Ryde, NSW, Australia) using a standard 1 hour processing cycle for embryonic kidneys and a standard 3 hour processing cycle for postnatal kidneys. Tissue was then embedded in liquid paraffin using stainless steel moulds.

E18.5 kidneys were serially sectioned at 4 µm, and all sections were mounted on Polysine coated slides. Approximately 250-300 sections were obtained from each E18.5 kidney. PN21 kidneys were exhaustively sectioned at 5 µm and every 40th and 42nd section was mounted on Polysine coated slides. Approximately 12-14 section pairs from a total of approximately 480-560 sections were collected from each PN21 kidney.

3.4.5.2 Histochemistry

Sections underwent histochemical staining for PNA as described in **Section 3.4.4.2.** Images of E15.5, E18.5 and PN21 kidney sections histochemically stained for PNA are presented in **Figure 3.3**.

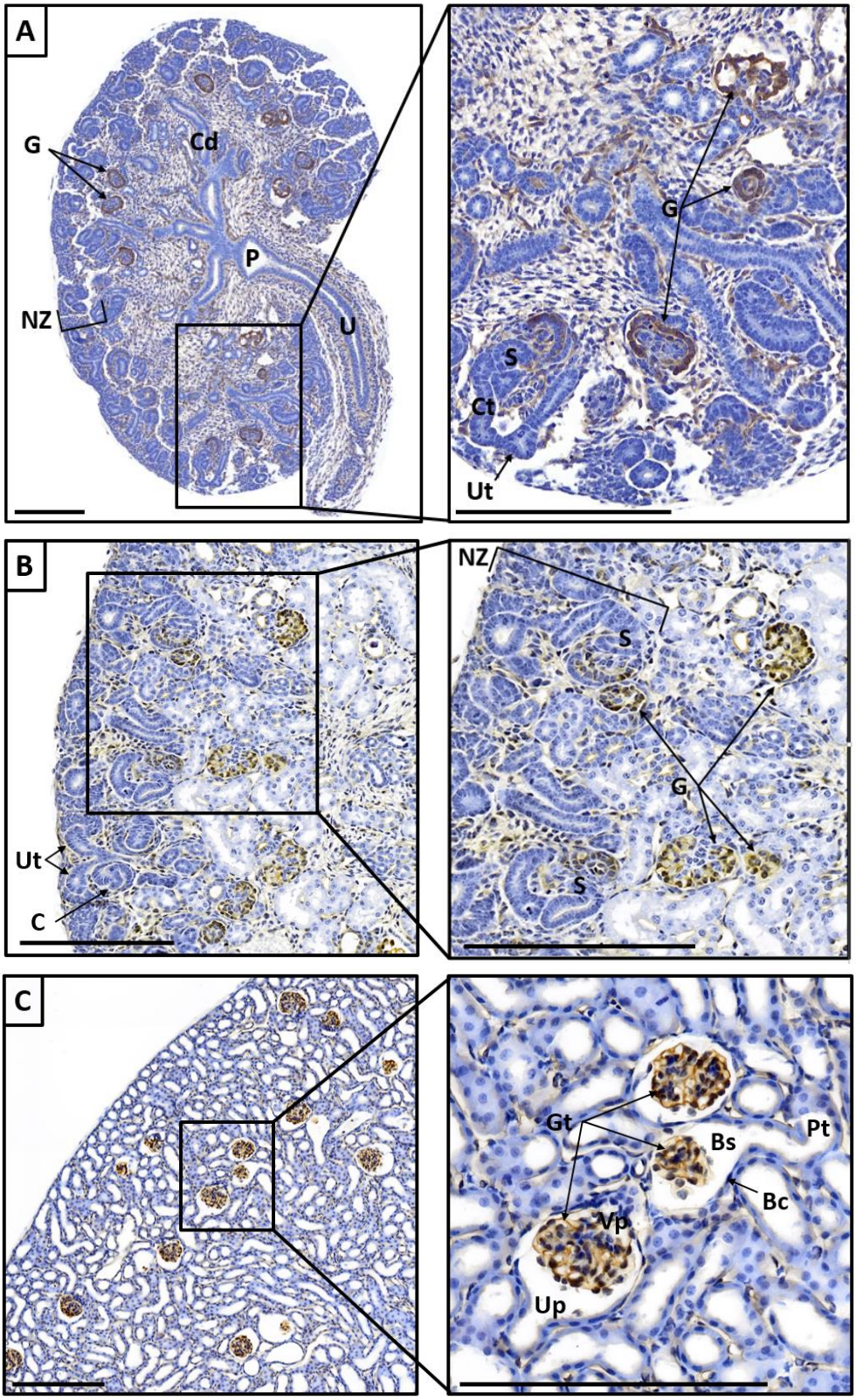


Figure 3.3 Histochemical staining of E15.5, E18.5 and PN21 kidneys

A E15.5, **B** E18.5 and **C** PN21 kidney sections histochemically stained for PNA, a marker of glomerular podocytes, and counterstained with Haematoxylin. Bc = Bowman's capsule, Bs = Bowman's space, C = comma-shaped body, Cd = collecting duct, Ct = connecting tubule, G = glomerulus, Gt = glomerular tuft, NZ = nephrogenic zone, P = pelvis, Pt = proximal tubule, S = S-shaped body, U = ureter, Up = urinary pole, Ut = ureteric tip, Vp = vascular pole. Scale bar = 200 µm.

3.4.5.3 Stereology

Total glomerular number was estimated using the physical disector/fractionator combination (Cullen-McEwen et al., 2012). This method estimates the total number of glomeruli within the kidney from data collected from the sampled pairs of sections; the sample section ('*n*') and the look-up section ('*n+2*').

3.4.5.3.1 Estimating glomerular number at E18.5

The total number of 4 µm sections from each E18.5 kidney was counted, and a section sampling fraction (SSF) determined so that approximately 10 section pairs would be analysed. Several factors need to be taken into consideration when determining the SSF. These include: (i) ensuring that no glomerulus is located between the two sections of a pair and is thereby missed; (ii) ensuring that section pairs are sufficiently separated from one another so that glomeruli are not seen in more than one pair and which reduces the time efficiency of the method; and (iii) ensuring that sufficient tissue is sampled so that at least 100 glomeruli are sampled using the disector principle and that therefore a precise estimate is obtained. To determine glomerular diameter and thereby SSF, approximately 30-35 glomeruli were tracked through serial sections from start to finish. Glomeruli at E18.5 were found to have an average diameter of approximately 50 µm. With approximately 250 to 300 sections obtained from each E18.5 kidney, a SSF of between 25 and 30 was used.

Section pairs were chosen with a random start in the sampling fraction. In a darkened room, using a light microscope (Olympus BX50) equipped with a projection arm, the entire first section of the section pair (the sample section) was projected onto A3 paper at a magnification of 150X (10X lens and 15X projection arm). The perimeter of the section was traced and PNA-positive glomeruli were circled. The second section of the pair (the look-up section) was then projected over the tracing of the sample section. The look-up section was assessed for glomeruli that were now absent compared to the sample section (described as a disappearing event). Newly appearing PNA-positive glomeruli were also circled and confirmed as an appearing event if absent in the sampled section. The total number of appearing and disappearing PNA-positive events were counted per section pair and tallied for each kidney. The total estimate of PNA-positive glomeruli in the kidney ($N_{glom,kid}$) was calculated using the following equation:

$$N_{glom,kid} = SSF \times ½ \times ½ \times Q^{-}$$

Where

1/SSF was the inverse of the SSF or the number of sections advanced between section pairs (in this case 25 or 30), the first ½ accounts for the number of sections that exist from *n* to *n*+2, the second ½

accounts for the fact that both appearing and disappearing PNA-positive events were counted, and Q^- was the total number of disappearing and appearing PNA-positive structures counted.

3.4.5.3.2 Estimating glomerular number at PN21

Total glomerular number was estimated in PN21 kidneys using the general approach used for the E18.5 kidneys (**Section 3.4.5.3.1**). However, due to the greater glomerular size, sections were analysed at a magnification of 60X (4X lens and 15X projection arm). Additionally, every 40th and 42nd section was collected (from a random start) and therefore the SSF was 40. A visual representation of the protocol used to estimate glomerular number in PN21 kidneys is presented in **Figure 3.4**.

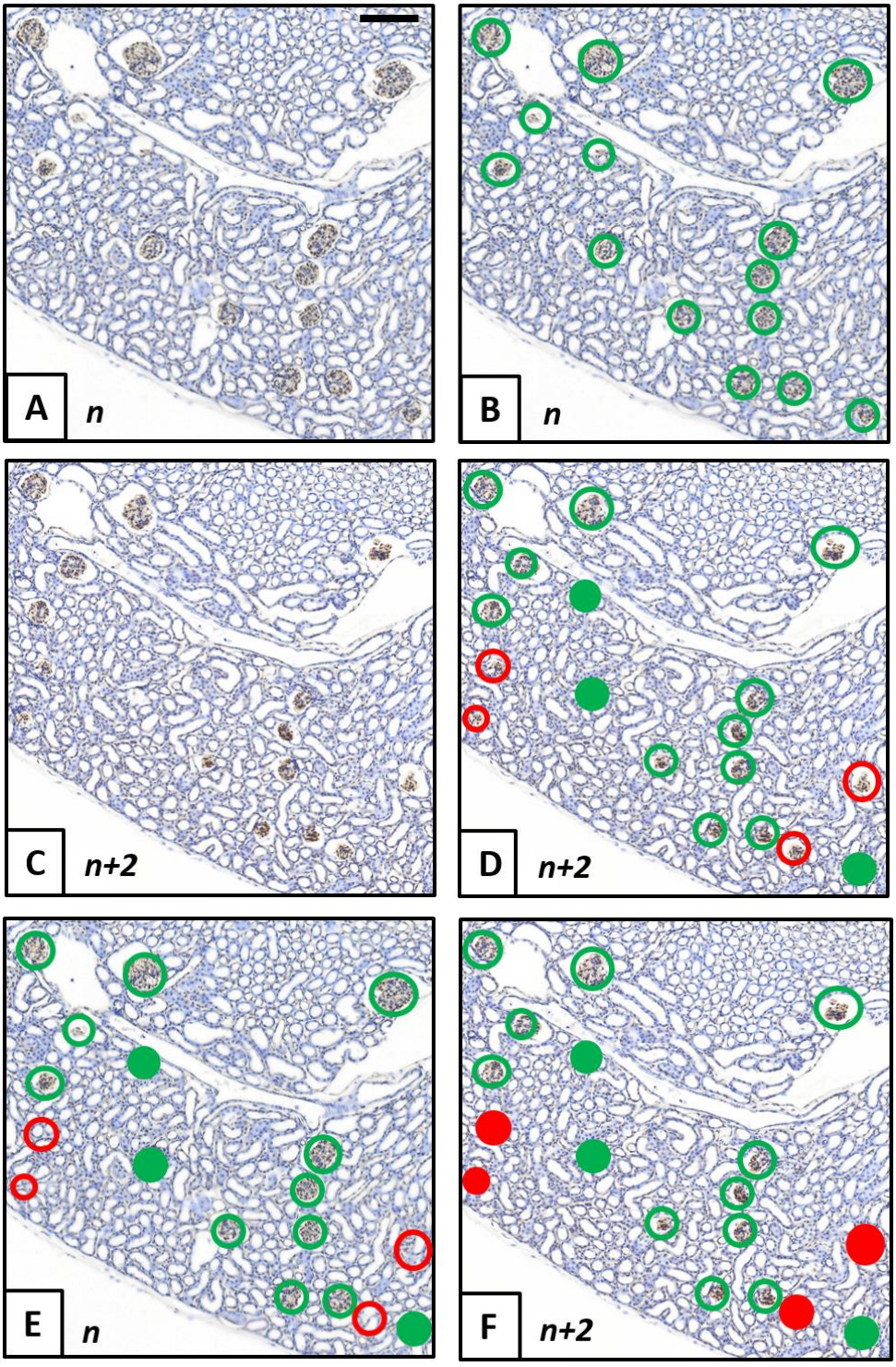


Figure 3.4 Estimating glomerular number in PN21 kidneys using PNA-lectin staining and the physical disector/fractionator combination.

PN21 kidney section pairs stained for PNA-lectin. **A** The sample section (*n*) is examined. **B** PNA-positive glomeruli in the sample section are outlined (green open circles). **C** The look-up section (*n+2*) is examined. **D** The trace from the sample section is overlaid on top of the look-up section. **E** Glomeruli present in the nth section but absent in the $n^{th}+2$ section (disappearing PNA-positive glomeruli) are marked (three full green circles). **F** Glomeruli present in the $n^{th}+2$ section but absent in the nth section (appearing PNA-positive glomeruli) are marked (four full red circles). This process is completed for the entire sample section and repeated for approximately ten pairs of tissue sections. Scale bar = 100 µm.

3.4.5.3.3 Estimating kidney volume at E18.5 and PN21

Kidney volume was estimated using the Cavalieri Principle. For E18.5 kidneys, the first section of the section pair (the sample section) was viewed on a projection light microscope and viewed at a final magnification of 60X. A stereological test grid (2x2 cm) was placed over the projected image and points falling on kidney tissue were counted. For PN21 kidneys, the first section of the section pair (the sample section) was placed on a microfiche reader and viewed at 24.25X. A stereological test grid (2x2 cm) was placed on the microfiche screen and points falling on kidney tissue were counted. Kidney volume was estimated using:

$$V_{kid} = \Sigma P \times a(P) \times T \times 1/SSF$$

Where

V_{kid} is kidney volume, ΣP is the total number of grid points overlaying the kidney tissue, a(P) is the area associated with each grid point at the relevant magnification, T is section thickness (4 µm for E18.5 kidneys; 5 µm for PN21 kidneys), and 1/SSF is the inverse of the SSF (25 or 30 for embryonic kidneys; 40 for postnatal kidneys).

3.4.5.3.4 Estimating mean and total glomerular volume at PN21

Mean glomerular volume and total glomerular volume (combined volume of all glomeruli in a kidney) were estimated in PN21 kidneys (Cullen-McEwen et al., 2003). The first section of every sampled section pair was placed on an Olympus BX50 light microscope fitted with a motorised stage (Autoscan Systems, Ormond, VIC, Australia). The section was projected onto a 2x2 cm counting grid (total of 64 grid points) in a darkened room at a final magnification of 300X (20X lens and 15X projection arm). The motorised stage was connected to a computer that controlled the stepping procedure for systematic uniform sampling (Oasis-blue Automation Controller, Version 3.2.1; Objective Imaging, Cambridge, UK). A step size of 1200 µm in the *x* and *y* axes was used. The number of grid points falling on kidney tissue (P_{kid}), glomerular tufts (P_{glom}) and renal corpuscles (P_{corp}) in each sampled area were counted. This was performed for each of the sample sections (every 40th).

Mean glomerular volume (V_{glom}) and mean renal corpuscle volume (V_{corp}) were estimated using the following formulae:

$$V_{glom} = [P_{glom}/P_{kid}]/[N_{glom,kid}/V_{kid}]$$

$$V_{corp} = [P_{corp}/P_{kid}]/[N_{glom,kid}/V_{kid}]$$

The combined volume of all glomeruli ($V_{glom,kid}$) and renal corpuscles ($V_{corp,kid}$) was estimated by multiplying the mean volumes by estimated total nephron number.

$$V_{glom,kid} = V_{glom} \times N_{glom,kid}$$

$$V_{corp,kid} = V_{corp} \times N_{glom,kid}$$

3.4.6 Assessment of metabolic health in adult offspring

A cohort of pups from high fat and normal fat fed dams were weaned onto standard animal chow at PN21 and kept into adulthood for assessment of metabolic function.

Offspring were weighed weekly from weaning until 9 months of age.

Body composition was assessed in offspring at 4, 6 and 9 months of age by DEXA, as described previously (**Section 2.4.5)**.

To assess if exposure to a high fat diet in development alters glucose handling, GTTs were performed in offspring at 4, 6 and 9 months of age. GTTs were performed as previously described (**Section 2.4.4**).

3.4.7 Assessment of renal function and albumin excretion in adult offspring

Renal function and albumin excretion were assessed in offspring to determine if exposure to a high fat diet during development has long-term implications for the kidney.

3.4.7.1 Renal function

There are several limitations in the techniques used to measure renal function in rodents. GFR can be determined by measuring creatinine clearance in blood and urine samples, yet is considered a poor marker of renal function as tubular secretion can account for up to 50% of total renal creatinine excretion in mice (Eisner et al., 2010). Methods that measure GFR by urinary clearance of radioactively labelled inulin are labour intensive as they require surgical preparation and constant infusion, and in addition require a terminal experiment. Furthermore, inulin clearance is measured under anaesthesia, which evidently confounds renal function considering the anaesthesia related decline in blood pressure and GFR.

Alternatively, renal function can be determined by the non-invasive transcutaneous measurement of fluorescein isothiocyanate (FITC)-sinistrin clearance. This technique, first described by Schock-Kusch et al. (2011) and Schreiber et al. (2012), allows real-time measurement of renal clearance in freely moving mice, independent of blood or urine sampling or laboratory assays. This technique measures renal clearance in conscious mice avoiding the confounding effects of anaesthesia, and also enables repeat measurements over time.

Sinistrin is an exogenous marker of renal function, used as a gold-standard for the measurement of human and veterinary GFR. Sinistrin is a plant-derived fructan that is not metabolised in blood, is neither reabsorbed nor secreted in the kidney, yet passes freely across the glomerular filtration barrier (Fresenius-Kabi, 2015). In order to measure sinistrin clearance in the mouse, FITC-labelled sinistrin is injected intravenously and the elimination kinetics (fluorescent signal) are detected transcutaneously by a miniaturised device. This miniaturised instrument is fitted with two light-emitting diodes (LED) with an emission maximum for FITC at 470 nm and a photodiode to detect the fluorescent light with a maximum sensitivity of 525 nm (Schreiber et al., 2012). Data sets are stored in an internal memory and can be downloaded to a computer for analysis of kinetic parameters.

Upon intravenous administration of FITC-sinistrin, the plasma concentration of sinistrin reaches a peak and subsequently diffuses from the vascular space into the interstitium. An equilibrium between the intravascular fluid and extravascular fluid is established within 10-15 minutes post-injection in C57BL6 mice. Following this, there is constant diffusion of sinistrin back to the intravascular fluid. The pharmacokinetics during this period (15-60 minutes post-injection) are dominated by renal excretion of sinistrin and represent a single exponential decay phase.

Previous publications concerning non-invasive transcutaneous measurement report the renal clearance of FITC-sinistrin (expressed as t half-life) and the calculation of GFR (expressed as µl/min/100g bodyweight; derived from t half-life and a semi-empirical conversion factor) (Schock-Kusch et al., 2009, Schreiber et al., 2012). Following personal communication with Professor Norbert Gretz (Head of the laboratory that developed the non-invasive transcutaneous method, University of Heidelberg, Mannheim, Germany), it has become apparent that calculating GFR based on a conversion factor should be avoided considering GFR is not calculated directly from the assessed data. Rather, the conversion factor was derived in a group of mice of uniform body weight and thus assumes comparable extracellular volumes and body weights between experimental groups, limiting its applicability in many studies. Moreover, the conversion to GFR is dependent on the administration of an exact dose of FITC-sinistrin, which, even with the most experienced animal technician, is rarely precise or consistent. Despite previous publications of the transcutaneous method reporting GFR calculated from a conversion factor, renal function in recent studies by the Gretz laboratory as well as in the present study is expressed as t half-life. T half-life represents the rate constant of the single exponential elimination phase of the fluorescence-time curve, and represents renal clearance of FITC-sinistrin as a parameter of renal function (Schock-Kusch et al., 2013).

Renal function was measured in offspring at 6 and 9 months of age. Mice were briefly anesthetised by exposure to 2.5% Isoflurane-oxygen gas, administered via a nose cone. A section of the back of the

mouse was shaved and depilated (Hair Removal Cream; Veet, Reckitt Benckiser, West Ryde, NSW, Australia). As the hair removal cream has fluorescent properties, mice were prepared the day prior to GFR measurement.

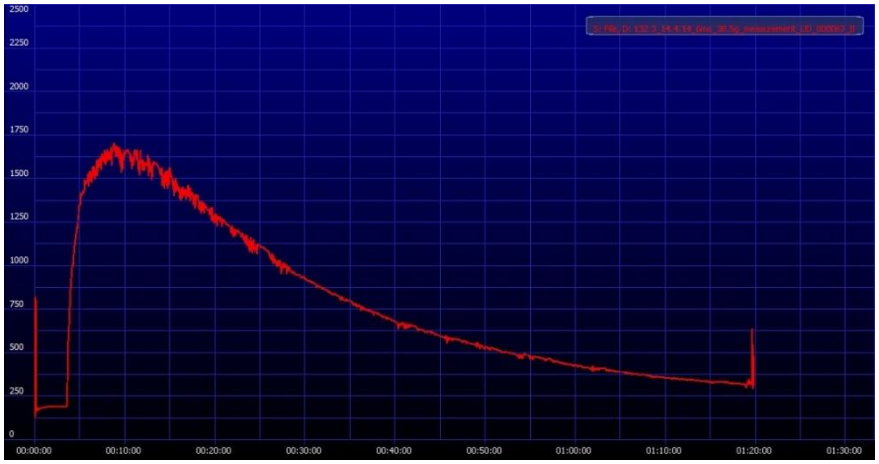
On the day of recording, mice were weighed, placed under a heat lamp for approximately 15 minutes to dilate the tail veins, and then anaesthetised. The miniaturised device was affixed to a double-sided adhesive patch (**Figure 3.5A**) and a small lithium polymer rechargeable battery was connected to the device, thus starting data acquisition (Mannheim Pharma & Diagnostics, Mannheim, Germany) (**Figure 3.5B**). The device was positioned on the depilated region of the mouse posterior to the scapulae and slightly lateral to the vertebral column to minimise movement artefacts and then secured with tape (Leukosilk; BSN Medical, Hamburg, Germany) (**Figure 3.5C**). Placement of LEDs on regions of skin with dark pigmentation was avoided. Background signal was measured for 2 minutes. FITC-sinistrin (Fresenius Kabi, Linz, Austria) was injected into the tail vein using a 30-gauge needle and syringe at a concentration of 12 mg/100 g body weight dissolved in sterile saline. Mice were removed from anaesthesia and kept in an individual cage (**Figure 3.5D**). FITC-sinistrin clearance was measured for at least 60 minutes, with a data acquisition period or measurement interval of 1.6 seconds (approximately 0.7 Hz). The device was then removed and mice were returned to their home cage.

Figure 3.5 Protocol for recording transcutaneous FITC-sinistrin clearance in adult offspring
A Mice were anaesthetised and the miniaturised device was affixed to double sided tape. **B** The battery was connected to the device to commence data acquisition and positioned on a depilated region of the mouse. **C** The device was secured with tape. Two minutes of background signal was recorded before mice were injected with FITC-sinstrin via the tail vein. **D** Mice were removed from anaesthesia and placed in a cage. FITC-sinistrin clearance was recorded for a minimum of 60 minutes before the device was removed.

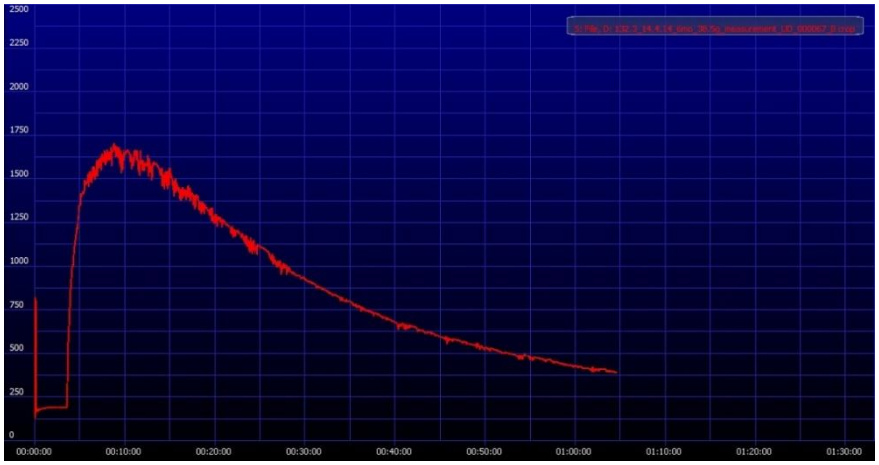
To visualise the recorded data, the device was connected to a computer via micro USB cable and read using software provided by Mannheim Pharma & Diagnostics (Sensor Control Application, version 15). Each data point (FITC signal) was plotted against time (**Figure 3.6A**) and saved as an Excel spreadsheet. Aberrant data points that arose during the removal of the device from the mouse were deleted from the end of the spreadsheet and the curve was reloaded (**Figure 3.6B**).

For data quantification, a one compartment, first order pharmacokinetics model was utilised. This is recommended for mouse measurements as the anaesthesia recovery phase can often cause movement artefacts in the first few minutes of the recording, which can result in bias. Using the Sensor Control Application software, the background signal of the measurement was marked as the 'offset' (**Figure 3.6C**), and a starting level, 15 minutes post injection (when an equilibrium between sinistrin in the intravascular and extravascular fluid is established, and the single exponential decay phase is reached), was marked as the 'start' (**Figure 3.6D**). This generates a coefficient of determination value to assess how well the data fit, as well as a t-half-life value based on the single exponential excretion phase of the excretion curve as a measure of renal function (**Figure 3.6E**).

A Raw data set. Y-axis = FITC-sinistrin signal. X-axis = time (minutes).

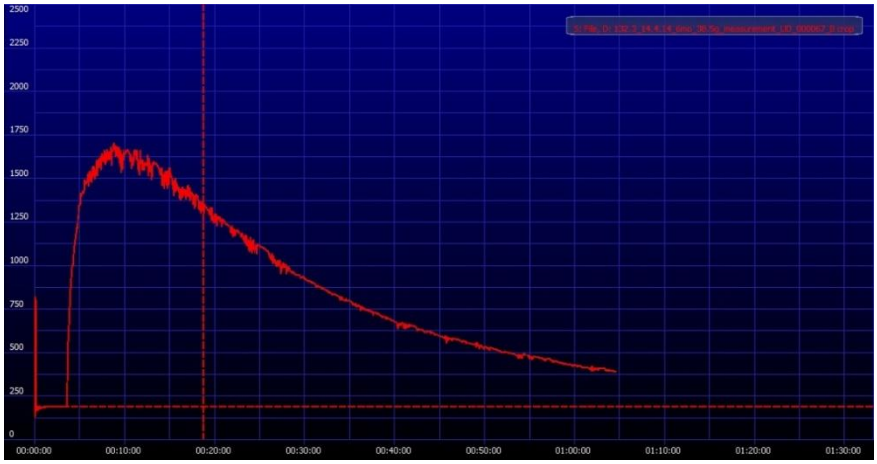


B Cropped data set. Erroneous values were deleted from the data set to remove aberrant signal during the removal of the device from the mouse and to give 60 minutes of recording.



C Based on the 2 minute background signal recorded prior to injection of FITC-sinistrin, a marker offset was specified (horizontal dashed red line).

D A marker start was specified 15 minutes after the injection of FITC-sinistrin (vertical dashed red line).



E Software statistics generate coefficient of determination (r^2 = 1.00), t-half-life ($t_{1/2}$ = 18:13 minutes) and upper and lower boundaries of the 95% confidence interval of the fit (CI min = 18:11, CI max = 18:15).

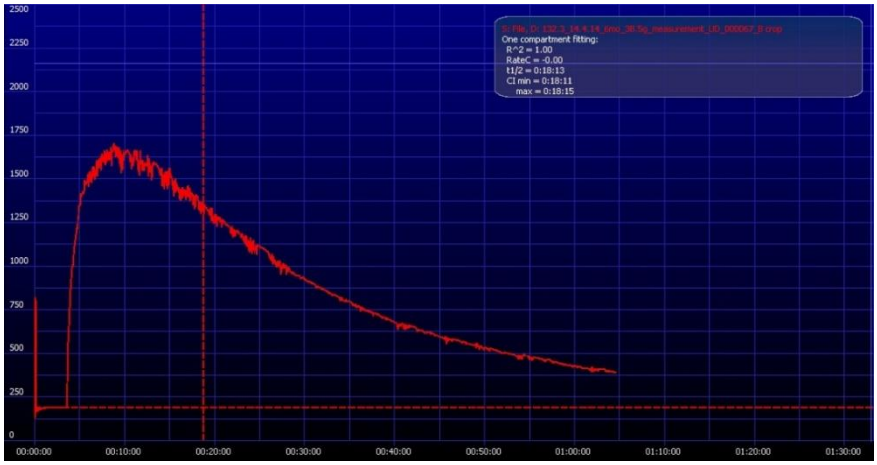


Figure 3.6 Data quantification to derive t half-life

A Visualisation, **B** cropping, and **C-E** data quantification of a FITC-sinistrin data set using a one-compartment model to derive t half-life.

3.4.7.2 Albumin excretion

Spot urine samples were collected from offspring at 9 months of age. This procedure involved holding mice over a plastic sheet and encouraging micturition by gentle trans-abdominal pressure over the bladder. Voided urine was aspirated into Eppendorf tubes and stored at -80°C. Approximately 50-200 µl of urine was collected. Urine collections were performed at the same time each day.

To assess urinary protein content, the albumin: creatinine ratio (µg/mg) was measured in spot urine using a murine microalbuminuria ELISA kit (Albuwell M, Exocell, Philadelphia, PA, USA) and Creatinine Companion (Exocell, Philadelphia, PA, USA). The urine albumin: creatinine ratio in a random urine sample was used as an alternative to the collection of cumbersome 24 hour urine samples and is well accepted for the detection of microalbumunuria (Warram et al., 1996).

3.4.7.2.1 Albumin ELISA

The microalbuminuria ELISA is an indirect competitive ELISA that employs a 96-well microplate coated with mouse albumin. To each well, 50 µl of murine serum albumin standard or urine sample were

added. Urine samples were used at a 1:5 dilution in NHE-BSA (stock diluent). 50 µl of rabbit anti-murine primary antibody was added to each well. Samples were incubated for 30 minutes at room temperature. Wells were washed ten times with enzyme immunoassay (EIA) buffer (0.15 M NaCl, 0.01 triethanolamine (pH 6.8) and 0.05% Tween 20). 100 µl of anti-rabbit HRP-conjugate was added to each well and incubated for 30 minutes at room temperature. Wells were subsequently washed ten times with EIA buffer before 100 µl of colour developer was added. Wells were incubated for 10 minutes before the reaction was stopped with 100 µl of 2 N sulphuric acid.

Colour intensity was measured on a microplate reader (SpectraMax 384 Microplate Reader, Molecular Devices, Sunnyvale, CA, USA) at 450 nm. Albumin concentrations were calculated from a semi-logarithmic plot of standard dilutions using Excel software (Microsoft Office, Redmond, WA, USA) to yield a mathematical model. Albumin concentrations were determined by taking the anti-log of the calculated values from this equation and multiplying by the inverse dilution factor. All samples were run in triplicate and a mean concentration value used in analysis. The coefficient of variation was 6.2%.

3.4.7.2.2 Creatinine assay

The Creatinine Companion utilises a chemical assay based on the Jaffe reaction of alkaline picrate with creatinine. To measure urine creatinine, 20 µl of creatinine standard or urine sample were added to each well of a 96-well microplate. Urine samples were used at a 1:10 dilution in water. 100 µl of picrate working solution (2 ml of 1 N NaOH mixed with 10 ml of picrate reagent) was added to each well. Samples were incubated for 10 minutes and absorbance was measured on a microplate reader (SpectraMax 384 Microplate Reader, Molecular Devices, Sunnyvale, CA, USA) at 500 nm. 50 µl of acid reagent was subsequently added to each well and the plate was incubated for 5 minutes. Absorbance was measured again at 500 nm. Differential absorbance of samples before and after the addition of acid corrects for colour generation that may arise due to interfering substances in the urine sample.

Using Excel software, a least squares regression line was determined from the delta absorbance of the creatinine standards. Sample creatinine concentrations were determined from delta absorbances using the regression line and were multiplied by the reciprocal of the dilution factor. The coefficient of variation was 10.0%.

The albumin: creatinine ratio (µg/mg) was determined by dividing the mean albumin concentration by the mean creatinine concentration for each urine sample.

3.4.8 Assessment of renal morphology in adult offspring

3.4.8.1 Tissue collection of 9 month old offspring

At 9 months of age, offspring were fasted for 6 hours and then perfusion fixed as described in **Section 3.4.1.2**. The left and right kidneys were then carefully excised, weighed and placed in formalin. Mesenteric, peri-renal and abdominal fat pads were also excised and weighed.

3.4.8.2 Tissue processing, sectioning and histochemistry

Left kidneys were transferred to 70% ethanol and processed to paraffin as outlined in **Section 3.4.5.1**. Paraffin blocks were serially sectioned at 5 µm, and every 60th and 62nd section was mounted on Polysine coated slides. Approximately 12-15 section pairs (from a total of approximately 720-900 sections) were collected per kidney. Sections were histochemically stained with PNA as described in **Section 3.4.4.2.**

3.4.8.3 Kidney volume, total glomerular volume and total renal corpuscle volume

Kidney volume was assessed in 9 month old offspring as described for PN21 kidneys in **Section 3.4.5.3.3**. Total glomerular and renal corpuscle volume in the kidney were estimated as previously described (Cullen-McEwen et al., 2003). P_{kid}, P_{glom}, and P_{corp} were determined in the first section of every sampled section pair of the kidney as described in **Section 3.4.5.3.4**. Total glomerular volume in the kidney ($V_{glom,kid}$) was then calculated by dividing the number of points overlying glomeruli by the number of points overlying kidney tissue (P_{glom}/P_{kid}) and then multiplying this kidney volume. A similar formula was used to estimate total renal corpuscle volume in the kidney ($V_{corp,kid}$).

$$V_{glom,kid} = [P_{glom}/P_{kid}] \times V_{kid}$$

$$V_{corp,kid} = [P_{corp}/P_{kid}] \times V_{kid}$$

3.4.9 Data analysis

Data were analysed using SPSS software and GraphPad Prism software. Measures of offspring growth prior to weaning, plasma content, kidney development, body composition and renal function were analysed by two-way ANOVA with maternal diet and offspring sex as main effects, and incorporating a mixed linear model to account for any intra-litter bias (Festing, 2006). Offspring glucose tolerance and body weight post weaning were analysed by repeated measures ANOVA with maternal diet and sex as main effects, using mean litter values. Spearman's rank coefficient with Bonferroni correction was used to measure associations between kidney development and variables of interest (offspring body weight, offspring kidney volume, maternal glucose AUC and maternal weight). Spearman's rank coefficient and multiple regression analysis were performed using STATA software. Throughout *n* refers to the number of dams or litters. Offspring sex was incorporated as a variable in

all analyses and had no statistically significant effect unless otherwise stated. Data were tested for normality using a D'Agostino-Pearson omnibus normality test. Data are presented as mean ± SEM. $P<0.05$ was considered statistically significant.

3.5 Results

3.5.1 Offspring growth and litter characteristics

3.5.1.1 Offspring growth and litter characteristics at E15.5

3.5.1.1.1 Similar litter size

At E15.5, litter size was comparable between NFD NGT and HFD IGT dams (NFD NGT 8.3 ± 0.4 embryos, HFD IGT 8.4 ± 0.5 embryos; *P*=0.866). The proportion of alive and resorbed embryos was similar between groups; 92% of embryos collected from NFD NGT dams were viable and 94% of embryos from HFD IGT dams were viable (**Figure 3.7**), with only one dead embryo observed in one litter of each diet. Data were analysed using the chi-squared statistic (2x3 contingency table) which did not show a significant difference between the two dietary groups (*P*=0.792).

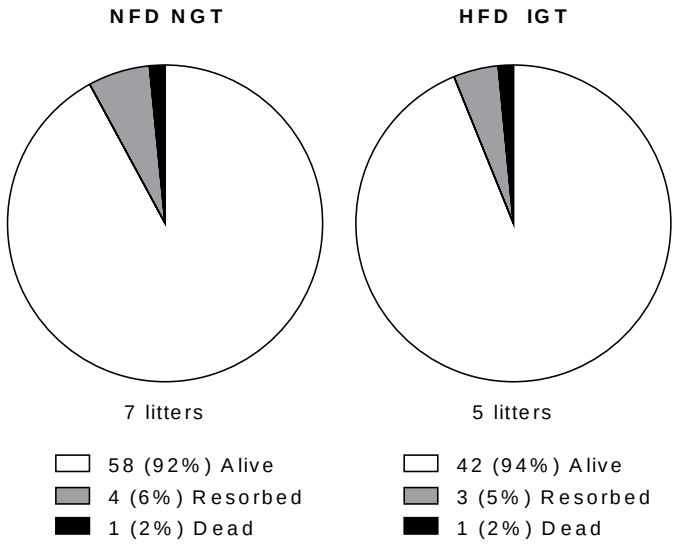


Figure 3.7 Litter characteristics at E15.5
Proportion of alive, resorbed and dead embryos in NFD NGT and HFD IGT litters at E15.5.

3.5.1.1.2 Similar uterus weight

At E15.5 there was no statistical difference in uterus weight between NFD NGT and HFD IGT dams (NFD NGT 6.9 ± 0.3 g, n=7; HFD IGT 7.0 ± 0.4 g, n=5; *P*=0.818).

3.5.1.1.3 Similar placental and embryo size

Values for growth parameters are provided in **Table 3.1**. There was no statistical difference in placental weight or body weight of embryos collected at E15.5. There was also no difference in the fetal: placental weight ratio, crown-rump length, or head diameter of embryos from NFD NGT and HFD IGT dams. Ponderal index (body weight/crown-rump length^3) was also similar in the two groups. Male

embryos were significantly heavier than female embryos at E15.5 (*P*=0.023). There was no difference in the ratio of male to female offspring (*P*=0.113). Values for these parameters are provided

Table 3.1 Growth parameters at E15.5

	Offspring of NFD NGT	Offspring of HFD IGT	P_{diet}	P_{sex}	$P_{diet*sex}$
Placental weight, mg	109.2 ± 3.3	108.6 ± 3.9	0.910	0.166	0.279
Body weight, mg	405.8 ± 9.7	402.7 ± 11.3	0.841	**0.023**	0.893
Fetal: placental weight ratio	3.74 ± 0.13	3.82 ± 0.15	0.744	0.977	0.449
Crown-rump length, mm	13.9 ± 0.2	13.6 ± 0.3	0.362	0.139	0.096
Head diameter, mm	7.9 ± 0.1	8.14 ± 0.2	0.334	0.451	0.609
Ponderal index, mg/mm^3	0.150 ± 0.002	0.155 ± 0.002	0.107	0.780	**0.032**

Analysis by two-way ANOVA incorporating maternal diet and sex, weighted for litter using a mixed linear model. Values are mean ± SEM. *n*(NFD NGT)= 56 embryos from 7 litters, *n*(HFD IGT)= 42 embryos from 5 litters.

3.5.1.2 Offspring growth and litter characteristics at E18.5

3.5.1.2.1 Similar litter size

As observed at E15.5, there was no difference in litter size between NFD NGT dams and HFD IGT dams at E18.5 (NFD NGT 9.1 ± 0.4 embryos, HFD IGT 8.4 ± 0.6 embryos; *P*=0.328). The proportion of alive and resorbed embryos was also similar in the two groups; 96% of embryos collected from NFD NGT dams were viable and 95% of embryos from HFD IGT dams were viable (**Figure 3.8**). No dead embryos were observed at E18.5. Data were analysed using the chi-squared test (2x2 contingency table) which did not show a significant difference between the two dietary groups (*P*=0.733).

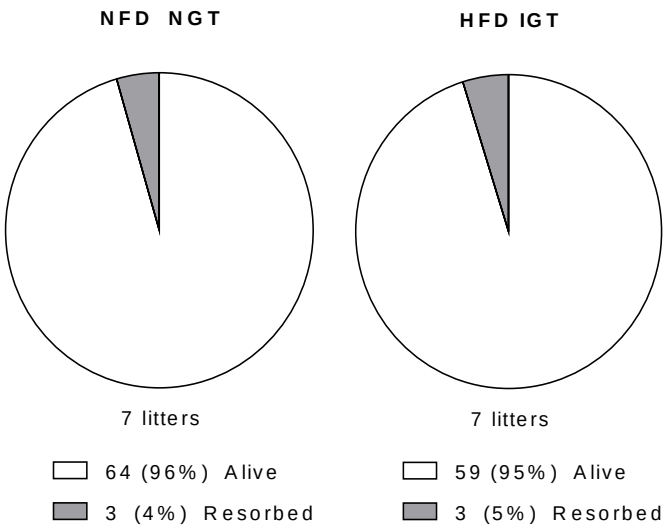


Figure 3.8 Litter characteristics at E18.5
Proportion of alive, resorbed and dead embryos in NFD NGT and HFD IGT litters at E18.5.

3.5.1.2.2 Similar uterus weight

Uterine horn weight was similar between groups at E18.5 (NFD NGT 12.9 ± 0.5 g, n=7; HFD IGT 12.4 ± 1.0 g, n=8; *P*=0.644).

3.5.1.2.3 Offspring of HFD IGT dams are macrosomic at E18.5

Values for growth parameters are provided in **Table 3.2**. Embryos of HFD IGT dams were macrosomic at E18.5, exhibiting a 6% greater body weight than embryos of NFD NGT dams (*P*=0.022). There was no difference in placental weight between the two dietary groups although male embryos had heavier placentae than female embryos (*P*=0.010). There was no difference in the fetal to placental weight ratio, crown-rump length, head diameter or ponderal index between the two groups at E18.5. There was also no difference in sex ratios at E18.5 (*P*=0.431).

Table 3.2 Growth parameters at E18.5

	Offspring of NFD NGT	Offspring of HFD IGT	P_{diet}	P_{sex}	$P_{diet*sex}$
Placental weight, mg	94.2 ± 6.1	111.1 ± 6.2	0.071	**0.010**	0.705
Body weight, mg	1076.7 ± 16.0	1139.3 ± 17.5	**0.022**	0.109	0.537
Fetal: placental weight ratio	11.7 ± 0.6	10.7 ± 0.6	0.271	0.088	0.910
Crown-rump length, mm	20.7 ± 0.2	20.9 ± 0.2	0.332	0.076	0.974
Head diameter, mm	11.2 ± 0.1	11.3 ± 0.1	0.445	0.535	0.310
Ponderal index, mg/mm^3	0.123 ± 0.003	0.126 ± 0.003	0.443	0.180	0.561

Analysis by two-way ANOVA incorporating maternal diet and sex, weighted for litter using a mixed linear model. Values are mean ± SEM. *n*(NFD NGT)=63 embryos from 7 litters, *n*(HFD IGT)=59 embryos from 7 litters.

3.5.1.2.4 Similar fetal plasma insulin and leptin

While HFD IGT dams were hyperinsulinaemic at E18.5, there was no significant difference in fetal plasma insulin levels between the two dietary groups (NFD NGT 1.49 ± 0.22 ng/ml, HFD IGT 2.02 ± 0.22 ng/ml; *P*=0.108, **Figure 3.9A**). Similarly, there was no difference in plasma leptin levels between offspring of NFD NGT and HFD IGT pregnancies at E18.5 (NFD NGT 0.41 ± 0.09 ng/ml, HFD IGT 0.37 ± 0.10 ng/ml; *P*=0.761, **Figure 3.9B**).

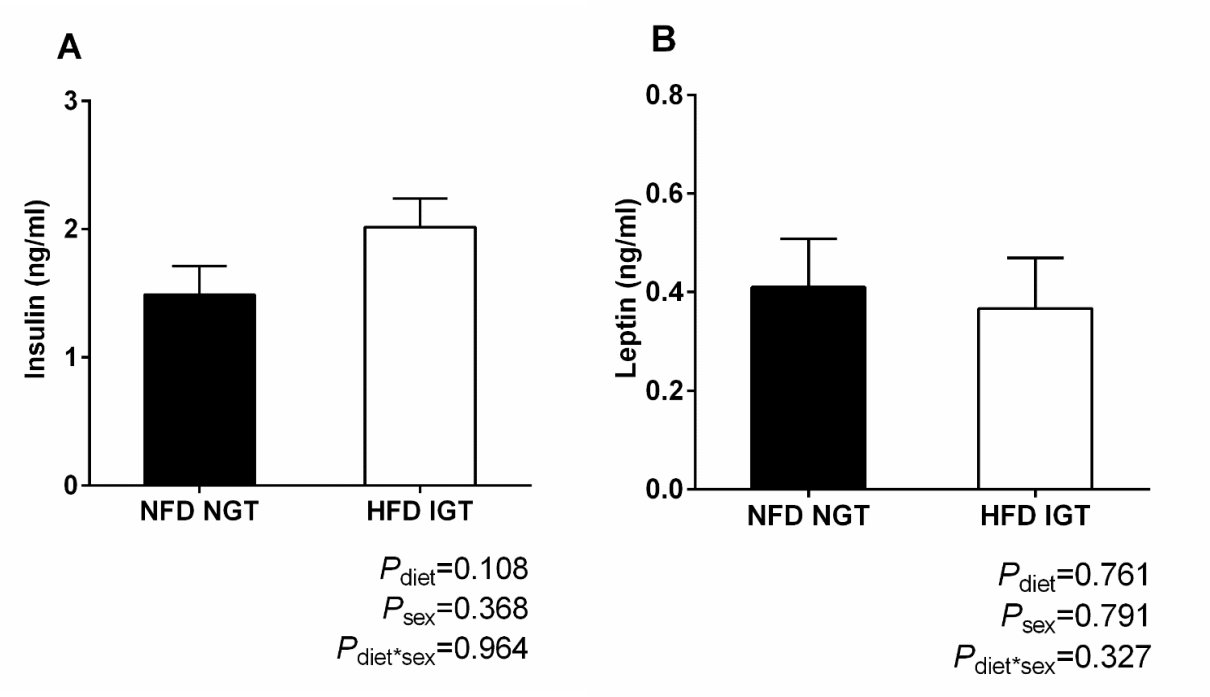


Figure 3.9 Fetal insulin and leptin levels at E18.5

A Fetal plasma insulin and **B** leptin levels in offspring of NFD NGT dams and HFD IGT dams. Analysis by two-way ANOVA, incorporating maternal diet and sex. Values are mean ± SEM. *n*(NFD NGT)=7 litters, *n*(HFD IGT)=8 litters.

3.5.1.3 Offspring growth and litter characteristics at PN21

3.5.1.3.1 Similar litter size

There was no difference in litter size between NFD NGT and HFD IGT dams at PN21 (NFD NGT 6.4 ± 0.5 pups, *n*=12 litters; HFD IGT 5.6 ± 0.6 pups, *n*=7 litters; *P*=0.288). Total litter weight was also comparable between groups at PN21 (NFD NGT 59.8 ± 4.6 g, *n*=12 litters; HFD IGT 58.0 ± 6.2 g, *n*=7 litters; *P*=0.812).

3.5.1.3.2 Similar offspring body weights during lactation

Offspring body weight was recorded between PN3 and PN21, however litters were unfortunately not weighed daily or on specific days and analysis by repeated measures ANOVA was therefore not feasible. Each time point was instead analysed separately by two-way ANOVA weighted for litter (**Table 3.3**). There was no difference in pup weights between PN3 and PN21 between the two groups, although there was a trend for offspring of HFD IGT dams to be heavier than offspring of NFD NGT dams at PN21 (*P*=0.065). Male offspring were significantly heavier than female offspring from PN6 onwards. There was no difference in the ratio of male to female offspring in the postnatal cohort (*P*=0.769).

Table 3.3 Offspring body weight in the early postnatal period

	Female of NFD NGT	Female of HFD IGT	Male of NFD NGT	Male of HFD IGT	*n*(NFD NGT) (litters)	*n*(HFD IGT) (litters)	P_{diet}	P_{sex}	$P_{diet*sex}$
PN3	1.96 ± 0.12	1.97 ± 0.14	2.01 ± 0.12	2.12 ± 0.14	9	7	0.736	0.083	0.340
PN6	2.80 ± 0.27	3.40 ± 0.34	3.02 ± 0.27	3.63 ± 0.34	8	5	0.184	**0.008**	0.931
PN9	4.45 ± 0.28	4.79 ± 0.42	4.70 ± 0.28	5.05 ± 0.42	11	5	0.500	**0.008**	0.976
PN12	5.83 ± 0.25	5.51 ± 0.36	6.03 ± 0.25	6.02 ± 0.35	8	4	0.704	**0.028**	0.295
PN15	7.20 ± 0.36	7.49 ± 0.41	7.55 ± 0.36	7.83 ± 0.41	9	7	0.603	**0.013**	0.999
PN18	7.96 ± 0.41	8.59 ± 0.41	8.20 ± 0.41	9.12 ± 0.41	7	7	0.194	**0.009**	0.311
PN21	9.46 ± 0.36	10.19 ± 0.48	10.09 ± 0.36	10.84 ± 0.48	12	7	0.065	**<0.0001**	0.824

Analysis at each postnatal day by two-way ANOVA incorporating maternal diet and sex, weighted for litter using a mixed linear model. Values are mean ± SEM.

3.5.1.3.3 Similar offspring body composition

Offspring body composition was assessed by DEXA at PN21. Representative images of DEXA scans in female PN21 offspring of NFD NGT and HFD IGT dams are presented in **Figure 3.10**. There was no difference in bone composition between maternal groups (**Table 3.4**). Offspring of HFD IGT dams had a slight increase in values for all body composition parameters however none of these were significantly different (**Table 3.4**). Male offspring had lower bone mineral content (P=0.019) but greater lean tissue mass (P<0.0001) and total tissue mass (P<0.0001) than female offspring.

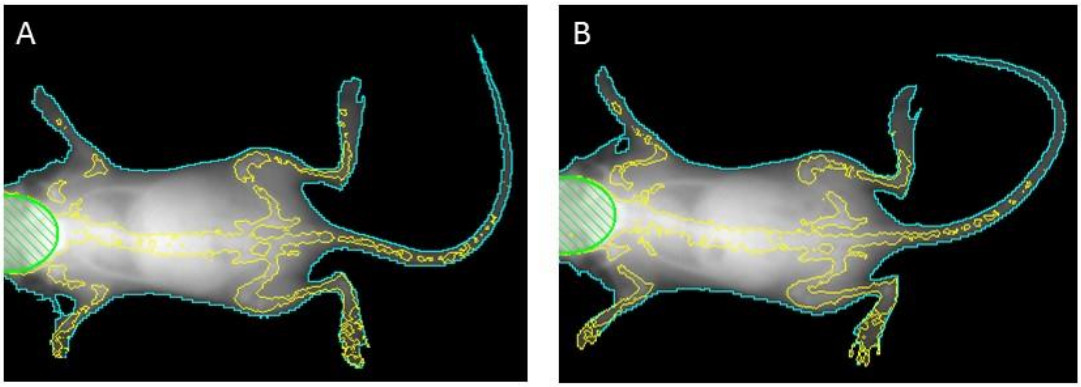


Figure 3.10 Representative DEXA scans of PN21 offspring
Representative DEXA scans of PN21 female offspring of **A** a NFD NGT dam and **B** a HFD IGT dam.

Table 3.4 Offspring bone and body composition at PN21

	Female of NFD NGT	Female of HFD IGT	Male of NFD NGT	Male of HFD IGT	P_{diet}	P_{sex}	$P_{diet*sex}$
Bone mineral density, g/cm^2	0.026 ± 0.001	0.028 ± 0.001	0.027 ± 0.001	0.029 ± 0.001	0.213	**0.019**	0.634
Bone mineral content, g	0.072 ± 0.007	0.088 ± 0.009	0.082 ± 0.008	0.093 ± 0.009	0.240	0.052	0.453
Bone area, m^2	2.69 ± 0.20	3.09 ± 0.23	2.98 ± 0.20	3.17 ± 0.23	0.357	0.077	0.290
Lean tissue, g	8.03 ± 0.28	8.47 ± 0.33	8.79 ± 0.29	9.10 ± 0.32	0.380	**<0.0001**	0.661
Fat tissue, g	1.64 ± 0.15	2.04 ± 0.17	1.75 ± 0.16	2.08 ± 0.17	0.117	0.381	0.677
Total tissue, g	9.68 ± 0.38	10.50 ± 0.44	10.55 ± 0.40	11.18 ± 0.44	0.217	**<0.0001**	0.613
Percent body fat, %	16.77 ± 0.97	19.15 ± 1.13	16.32 ± 1.01	18.35 ± 1.12	0.167	0.378	0.527

Analysis by two-way ANOVA incorporating maternal diet and sex, weighted for litter using mixed linear model. Values are mean ± SEM. *n*(NFD NGT female)=18 offspring from 9 litters, *n*(NFD NGT male)=15 offspring from 6 litters, *n*(HFD IGT female)= 12 offspring from 7 litters, *n*(HFD IGT male)= 13 offspring from 7 litters

3.5.1.3.4 Similar offspring kidney weight

As shown in **Table 3.5**, there was no significant difference in kidney weight or the kidney weight: body weight ratio between the two dietary groups at PN21. A random blood glucose value was also taken at PN21 and was similar between pups from NFD NGT and HFD IGT dams (**Table 3.5**).

Table 3.5 Offspring kidney weights and blood glucose levels at PN21

	Offspring of NFD NGT	Offspring of HFD IGT	P_{diet}	P_{sex}	$P_{diet*sex}$
Right kidney weight, mg	86.7 ± 3.0	94.6 ± 4.3	0.161	0.440	0.812
Left kidney weight, mg	83.6 ± 3.8	85.2 ± 5.3	0.816	0.107	0.917
Total kidney weight, mg	170.6 ± 8.0	180.9 ± 9.8	0.404	0.281	0.997
Total kidney weight: body weight ratio	175.6 ± 4.6	170.3 ± 6.6	0.524	0.488	0.448
Random blood glucose, mmol/l	12.3 ± 0.8	13.6 ± 1.3	0.420	0.902	0.391

Analysis by two-way ANOVA incorporating maternal diet and sex, weighted for litter using a mixed linear model. Values are mean ± SEM. *n*(NFD NGT)=31 pups from 11 litters, *n*(HFD IGT)=18 pups from 6 litters.

3.5.2 Offspring kidney development

3.5.2.1 Normal ureteric tree development at E15.5

The ureteric trees from one male and one female embryo of average body weight from each litter were analysed at E15.5. Representative images of rendered 3D OPT datasets of kidneys of offspring of NFD NGT and HFD IGT dams are presented in **Figure 3.11**.

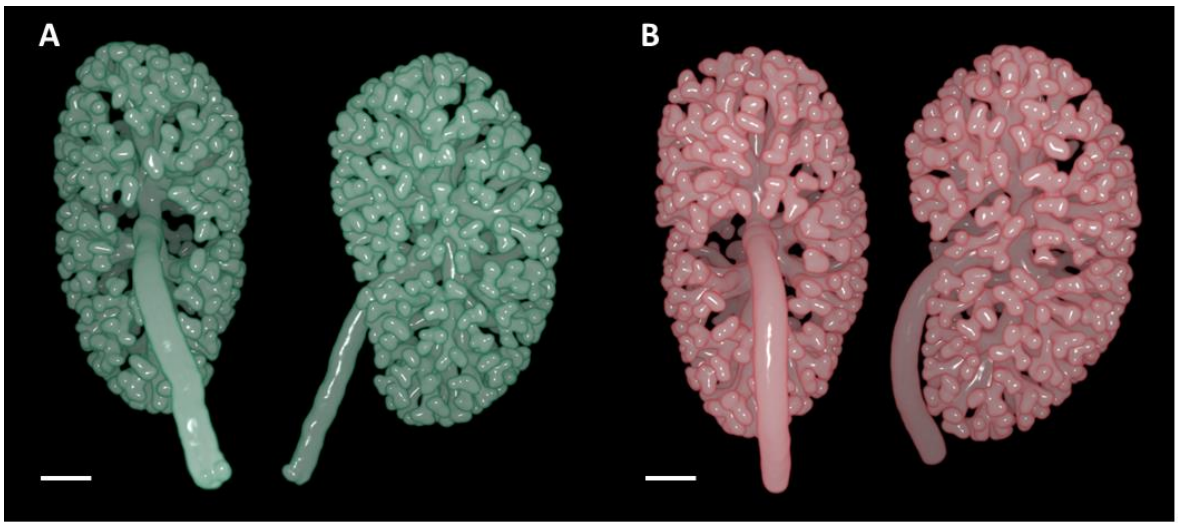


Figure 3.11 Images of 3D OPT datasets rendered using Drishti software.
Representative kidney of offspring of **A** NFD NGT and **B** HFD IGT. Scale bar = 200 µm.

Overall, there was no effect of maternal diet on offspring ureteric tree development at E15.5. As shown in **Figure 3.12A-D**, there was no difference in ureteric tip number (NFD NGT 392 ± 15 tips, HFD IGT 409 ± 17 tips; *P*=0.456), branch points (NFD NGT 389 ± 15 points, HFD IGT 406 ± 17 points; *P*=0.465), total ureteric tree length (NFD NGT 55,529.4 ± 2617.5 µm, HFD IGT 56,636.1 ± 2867.4 µm; *P*=0.779) or total ureteric tree volume (NFD NGT 92.0 ± 7.3 $\mu m^3 \times 10^6$, HFD IGT 83.3 ± 8.0 $\mu m^3 \times 10^6$; *P*=0.435) between offspring of NFD NGT and HFD IGT dams. When expressed relative to body weight, there was no difference in ureteric tree development (data not shown). Ureteric tip length and branch segment length were also similar between the two groups (**Table 3.6**). Males were found to have greater ureteric tree volume (*P*=0.014) and ureteric tip length (*P*<0.0001) than females (**Figure 3.12D** and **Table 3.6**). There was no difference in the total number of branch generations between kidneys of offspring of NFD NGT and HFD IGT dams (**Table 3.6**).

Table 3.6 Parameters of ureteric tree development of E15.5 offspring

	Offspring of NFD NGT	**Offspring of HFD IGT**	P_{diet}	P_{sex}	$P_{diet*sex}$
Ureteric tip length, µm	66.9 ± 1.7	65.1 ± 1.8	0.505	**<0.0001**	**0.027**
Branch segment length, µm	74.9 ± 1.3	75.9 ± 1.4	0.607	0.458	0.312
Number of branch generations	11.9 ± 0.4	12.6 ± 0.4	0.231	0.835	0.613

Analysis by two-way ANOVA incorporating maternal diet and sex, weighted for litter using a mixed linear model. Values are mean ± SEM. *n*(NFD NGT)=12 kidneys from 6 litters, *n*(HFD IGT)=10 kidneys from 5 litters.

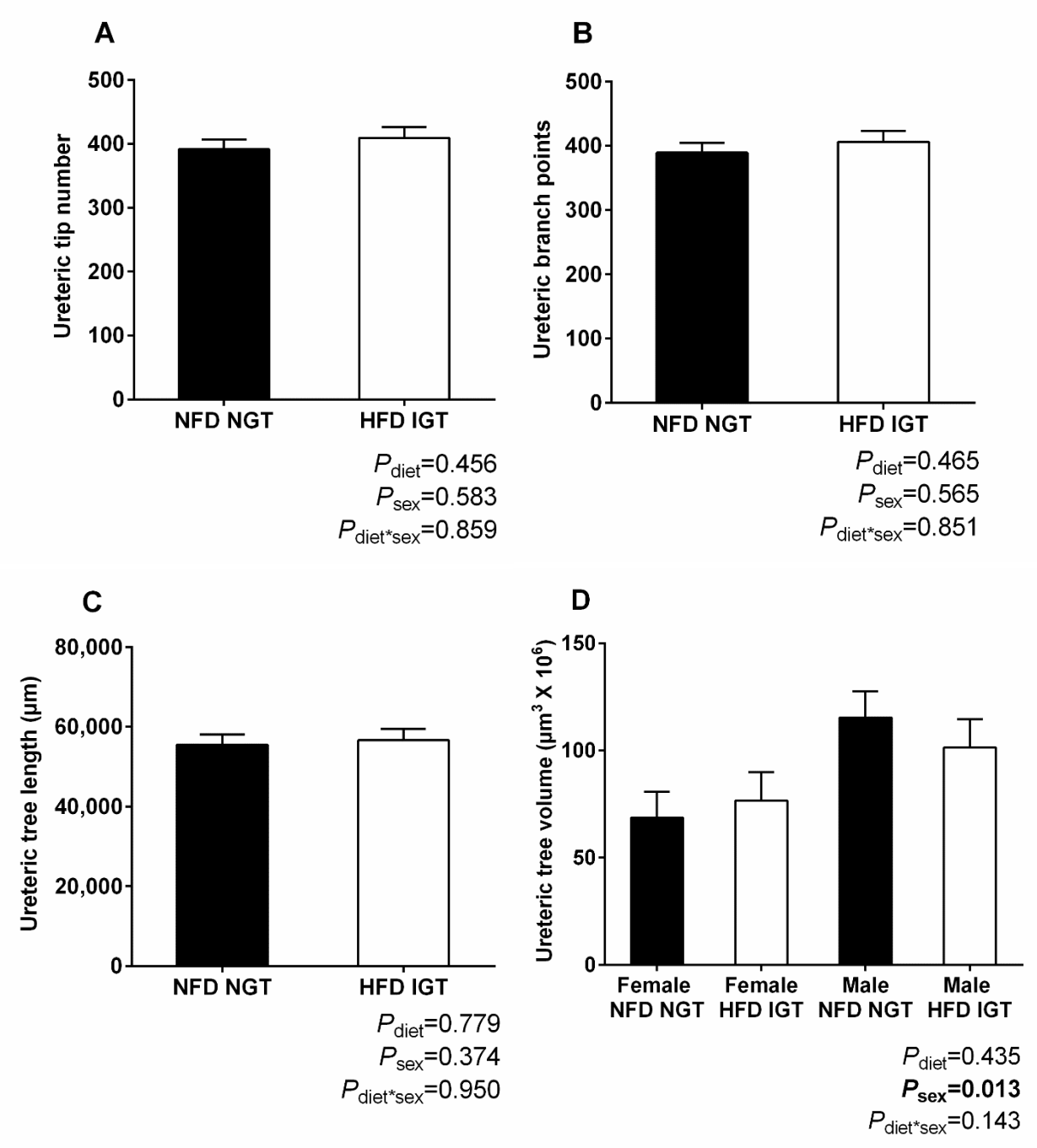


Figure 3.12 Ureteric tip number, branch length, tree length and tree volume at E15.5
Ureteric **A** tip number, **B** branch points, **C** tree length and **D** tree volume in E15.5 offspring of normal and high fat fed dams. Analysis by two-way ANOVA incorporating maternal diet and sex at each time point, weighted for litter using a mixed linear model. Values are mean ± SEM. *n*(NFD NGT)=12 kidneys from 6 litters, *n*(HFD IGT)=10 kidneys from 5 litters.

3.5.2.2 Normal total nephron number at E15.5

Offspring of NFD NGT and HFD IGT dams had a similar nephron number at E15.5 (NFD NGT 172 ± 12 nephrons, HFD IGT 179 ± 13 nephrons; *P*=0.735, **Figure 3.13**). There was no difference in the nephron number to body weight ratio (NFD NGT 419 ± 21 nephrons/g, HFD IGT 439 ± 22 nephrons/g; *P*=0.511).

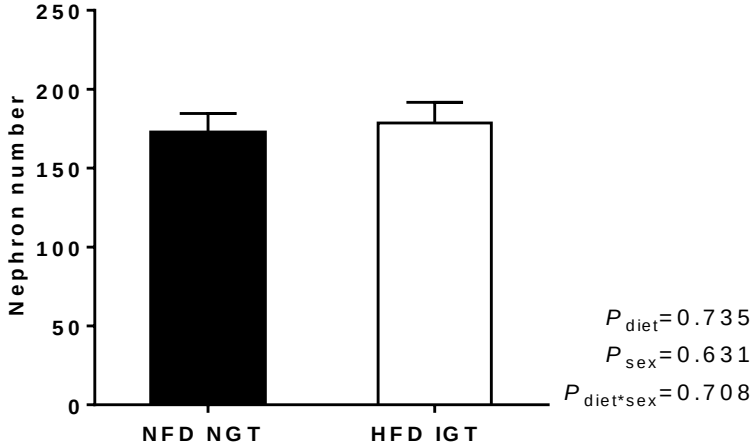


Figure 3.13 Offspring nephron number at E15.5
Total nephron number of NFD NGT dams and HFD IGT dams at E15.5. Analysis by two-way ANOVA for maternal diet and offspring sex, weighted for litter using a mixed linear model. Values are mean ± SEM. *n*(NFD NGT)=11 kidneys from 6 litters, *n*(HFD IGT)=10 kidneys from 5 litters.

3.5.2.3 Increase in early nephron number in offspring of HFD IGT dams at E18.5

Early nephron number was also assessed at E18.5. Offspring of HFD IGT dams had 25% more nephrons than offspring of NFD NGT dams (NFD NGT 1358 ± 53 nephrons, HFD IGT 1699 ± 58 nephrons; *P*=0.001, **Figure 3.14A**). When assessed relative to body weight, offspring of HFD IGT dams had significantly more nephrons per gram of body weight at E18.5 (NFD NGT 1239 ± 37 nephrons/g, HFD IGT 1478 ± 42 nephrons/g; *P*<0.0001, **Figure 3.14B**). Kidney volume was also greater by 24% in offspring of HFD IGT dams compared with offspring of NFD NGT dams (NFD NGT 1.84 ± 0.09 mm^3, HFD IGT 2.28 ± 0.09 mm^3; *P*=0.012, **Figure 3.14C**) and this increase was maintained when adjusted for body weight (NFD NGT 1.69 ± 0.06 mm^3/g, HFD IGT 1.99 ± 0.07 mm^3/g; *P*=0.003, **Figure 3.14D**). There was no difference in kidney development between male and female offspring at E18.5. Representative images of sectioned E18.5 kidneys of offspring of NGT NGT and HFD IGT dams are presented in **Figure 3.15**.

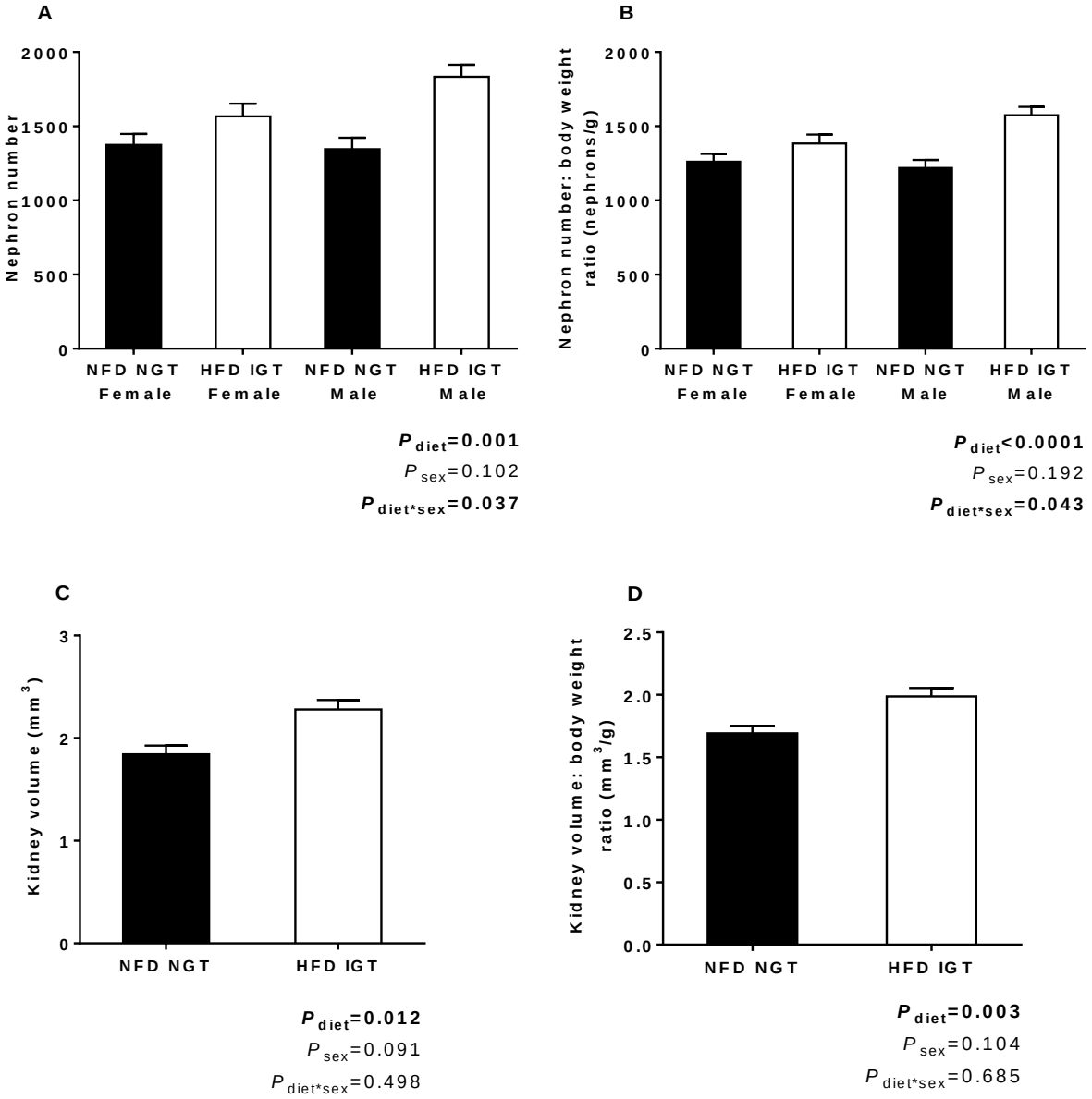


Figure 3.14 Offspring nephron number and kidney volume at E18.5

A Total nephron number, **B** nephron number: body weight ratio, **C** kidney volume and **D** kidney volume: body weight ratio of offspring of NFD NGT dams and HFD IGT dams at E18.5. Analysis by two-way ANOVA for maternal diet and offspring sex, weighted for litter using a mixed linear model. Values are mean ± SEM. (*n*)NFD NGT female=11 kidneys from 7 litters, (*n*)NFD NGT male=10 kidneys from 5 litters, (*n*)HFD IGT female=8 kidneys from 6 litters, (*n*)HFD IGT male=9 kidneys from 6 litters.

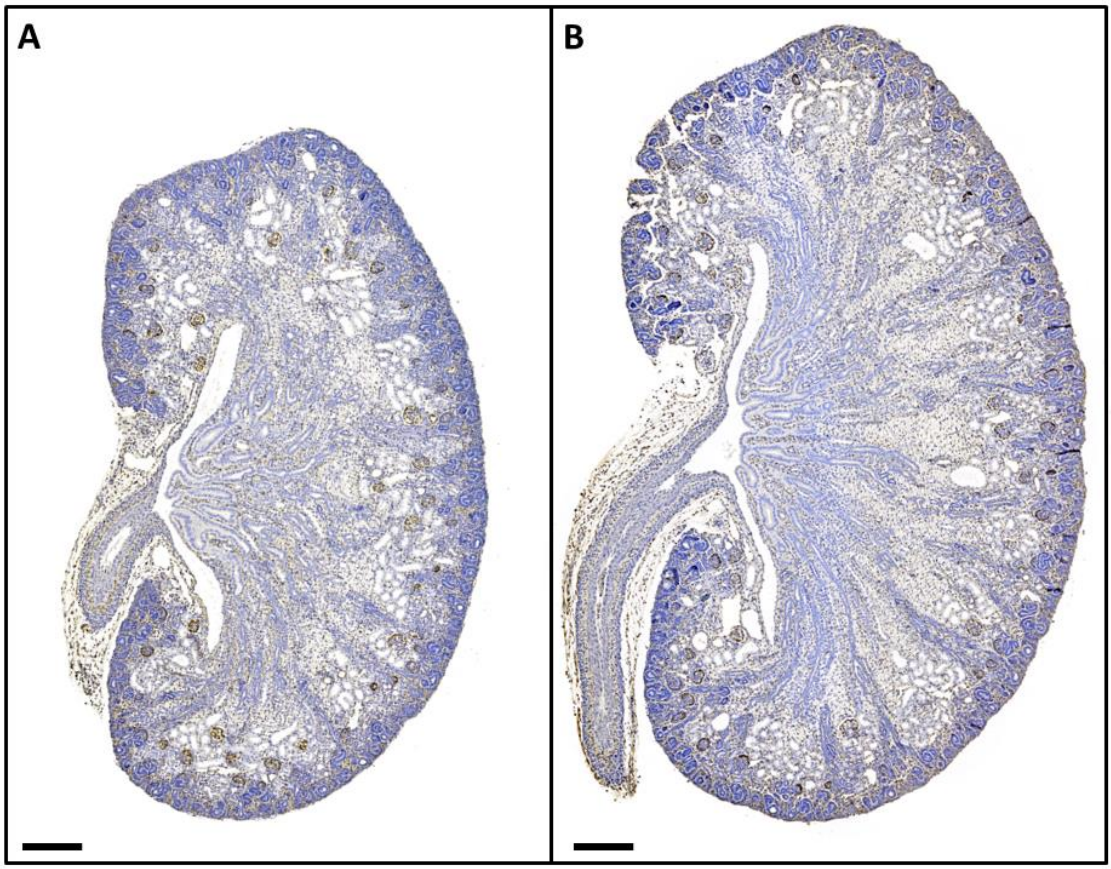


Figure 3.15 Representative images of E18.5 kidney sections
Representative images of sectioned E18.5 kidneys from offspring of **A** NFD NGT and **B** HFD IGT dams. Sections were histochemically stained for PNA and counterstained with Haematoxylin. Scale bar = 200 µm.

3.5.2.4 Increased nephron endowment in offspring of HFD IGT dams at PN21

At weaning (PN21), the kidneys of offspring of HFD IGT dams contained 20% more nephrons than offspring of NFD NGT dams (NFD NGT 15,431 ± 412 nephrons, HFD IGT 18,548 ± 458 nephrons; $P<0.0001$; **Figure 3.16A**). This increase is similar to the 25% increase in nephron number observed at E18.5. Nephron endowment was also assessed relative to body weight at PN21: offspring of HFD IGT dams had 14% more nephrons per gram body weight than offspring of NFD NGT dams (NFD NGT 1,626 ± 60 nephrons/g, HFD IGT 1,856 ± 70 nephrons/g; $P=0.028$; **Figure 3.16B**). Kidney volume was 18% greater in offspring of HFD IGT dams (NFD NGT 43.1 ± 1.9 mm^3, HFD IGT 50.9 ± 2.2 mm^3; $P=0.014$; **Figure 3.16C**) and there was a trend for kidney volume to be greater when expressed relative to body weight (NFD NGT 4.53 ± 0.17 mm^3/g, HFD IGT 50.60 ± 0.20 mm^3/g; $P=0.061$; **Figure 3.16D**).

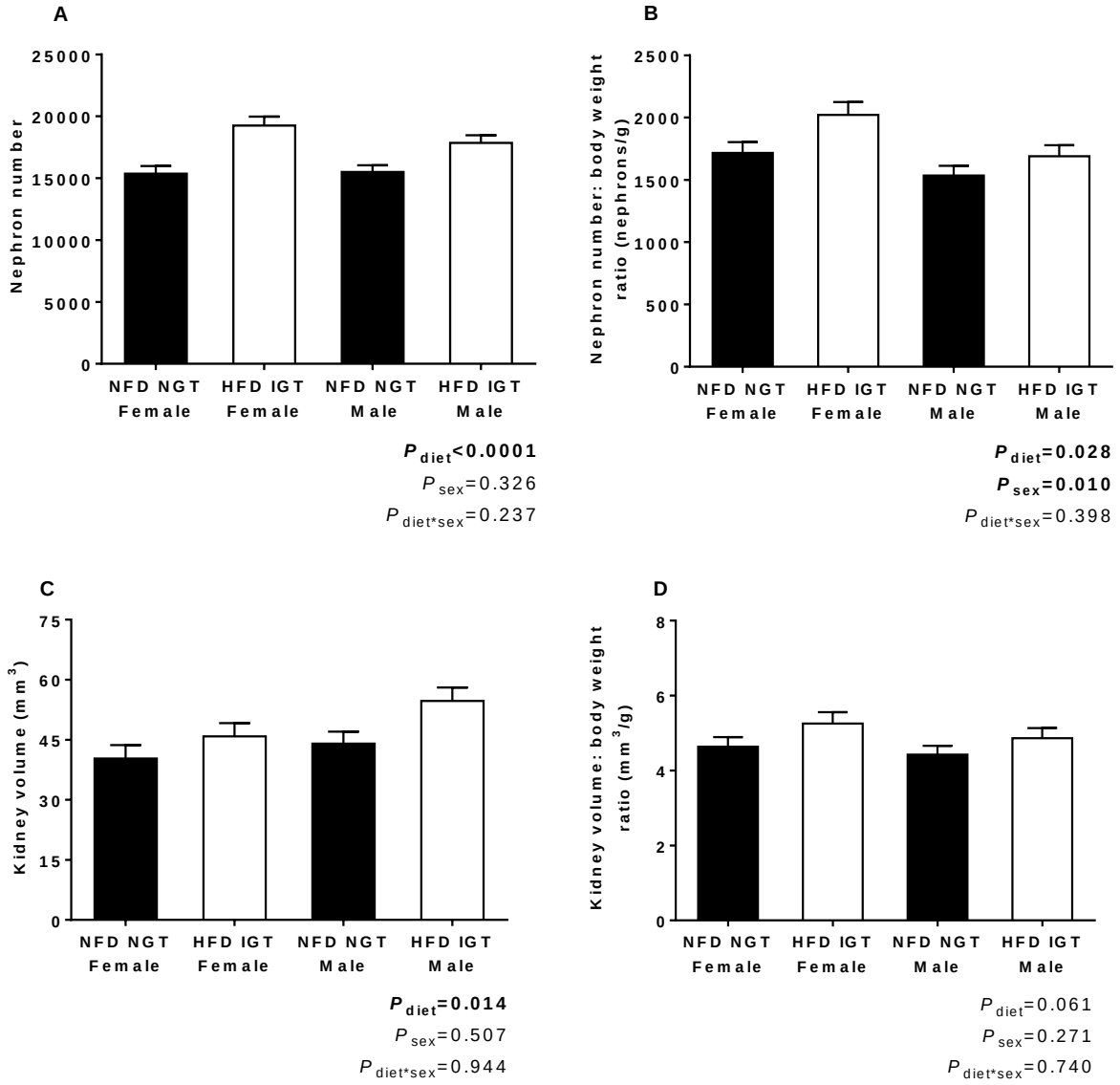


Figure 3.16 Offspring nephron endowment and kidney volume at PN21

A Nephron endowment, **B** nephron endowment: body weight ratio, **C** kidney volume and **D** kidney volume: body weight ratio of offspring of NFD NGT dams and HFD IGT dams at PN21. Analysis by two-way ANOVA for maternal diet and offspring sex, weighted for litter using a mixed linear model. Values are mean ± SEM. (*n*)NFD NGT female=6 kidneys from 6 litters, (*n*)NFD NGT male=8 kidneys from 8 litters, (*n*)HFD IGT female=6 kidneys from 6 litters, (*n*)HFD IGT male=6 kidneys from 6 litters.

Mean and total glomerular and renal corpuscle volumes are presented in **Table 3.7**. There was no difference in mean glomerular volume or mean renal corpuscle volume between groups. Despite a 20% greater nephron endowment, total glomerular volume was comparable in offspring of HFD IGT dams and offspring of NFD NGT dams. Total renal corpuscle volume was significantly elevated in offspring of HFD IGT dams (*P*=0.017). Relative to body weight, total glomerular volume and total renal corpuscle volume were similar in the two groups. Male offspring had greater mean glomerular volume (*P*=0.035) and mean corpuscle volumes (*P*=0.034) than female offspring.

Table 3.7 Mean and total glomerular and renal corpuscle volume of PN21 offspring

	Offspring of NFD NGT	Offspring of HFD IGT	P_{diet}	P_{sex}	$P_{diet*sex}$
Mean glomerular volume, V_{glom}, x10^{-4} mm^3	0.635 ± 0.030	0.586 ± 0.035	0.319	**0.035**	0.409
Mean renal corpuscle volume, V_{corp}, x10^{-4} mm^3	0.929 ± 0.038	0.932 ± 0.045	0.965	**0.034**	0.867
Total glomerular volume, $V_{glom,kid}$, mm^3	0.969 ± 0.054	1.082 ± 0.062	0.195	0.090	0.550
Total corpuscle volume, $V_{corp,kid}$, mm^3	1.425 ± 0.066	1.712 ± 0.078	**0.017**	0.178	0.689
Total glomerular volume: body weight ratio, $V_{glom,kid}$, mm^3/g	0.102 ± 0.004	0.107 ± 0.005	0.482	0.597	0.576
Total renal corpuscle volume: body weight ratio, $V_{corp,kid}$, mm^3/g	0.150 ± 0.006	0.171 ± 0.007	0.068	0.883	0.537

Analysis by two-way ANOVA incorporating maternal diet and sex, weighted for litter using a mixed linear model. Values are mean ± SEM. *n*(NFD NGT)=16 kidneys from 8 litters, *n*(HFD IGT)=12 kidneys from 6 litters.

3.5.2.4.1 Increased nephron endowment in offspring of high fat fed dams with normal glucose tolerance

In order to define the role of maternal high fat feeding and maternal glucose intolerance on offspring kidney development, nephron endowment was assessed in offspring of high fat fed dams with normal glucose tolerance prior to pregnancy (maternal glucose AUC pre-pregnancy < 1598 mmol/l.min, abbreviated as HFD NGT). As shown in **Figure 3.17**, nephron endowment was similar in offspring of HFD NGT and HFD IGT dams (HFD NGT 18,493 ± 478 nephrons, HFD IGT 18,548 ± 485 nephrons; *P*=0.937), with offspring of HFD NGT dams having, on average, 20% more nephrons per kidney than offspring of NFD NGT dams (NFD NGT 15,431 ± 412 nephrons; *P*<0.0001). These data indicate that high fat feeding has a greater influence on offspring nephron endowment than maternal glucose levels. It is worth noting that there was no difference in body weight between offspring of HFD NGT and HFD IGT dams at E18.5 (HFD NGT 1077.3 ± 33.1 mg, *n*=4 litters, HFD IGT 1139.0 ± 27.2 mg, *n*=7 litters; *P*=0.184). Similarly, body weights were similar between HFD NGT and HFD IGT offspring at PN3 (HFD NGT 2.26 ± 0.15 g, *n*=8 litters, HFD IGT 2.05 ± 0.17 g, *n*=6 litters; *P*=0.390) and PN21 (HFD NGT 10.43 ± 0.60 g, *n*=8 litters, HFD IGT 10.89 ± 0.69 g, *n*=6 litters; *P*=0.625).

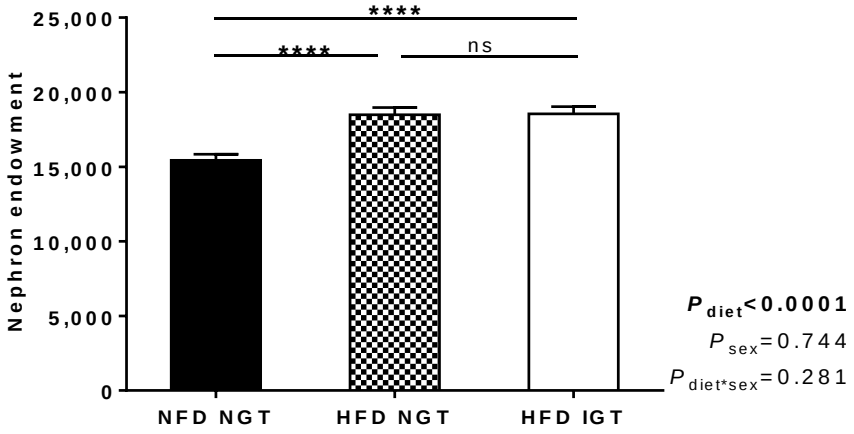


Figure 3.17 Nephron endowment in offspring of high fat fed dams with normal and abnormal glucose tolerance

Nephron endowment in PN21 offspring of NFD NGT, HFD NGT and HFD IGT dams. Analysis by two-way ANOVA for maternal diet and offspring sex, weighted for litter using a mixed linear model. Post-hoc analysis by Fisher's least significant difference. Values are mean ± SEM. *n*(NFD NGT)=16 kidneys from 8 litters, *n*(HFD NGT)=12 kidneys from 7 litters, *n*(HFD IGT)=12 kidneys from 6 litters. ****P<0.0001. NFD NGT=normal fat diet with normal glucose tolerance, HFD IGT=high fat diet with impaired glucose tolerance, HFD NGT=high fat fed diet with normal glucose tolerance.

3.5.2.4.2 Increased nephron endowment in offspring of high fat fed dams with normal body weight

Offspring nephron number was also assessed in dams based on their body weight prior to pregnancy. HFD mice were divided into two groups: those with body weight prior to pregnancy less than one SD above the mean value for NFD mice (< 24.7g, abbreviated as HFD NBW); and those with body weight prior to pregnancy greater than one SD above the mean value for NFD mice (> 24.7g, abbreviated as HFD HBW). Only NFD mice with body weights within one SD of the mean were included in the analysis (abbreviated as NFD NBW). As shown in **Figure 3.18**, nephron endowment was similar in offspring of HFD NBW and HFD HBW dams (HFD NBW 18,072 ± 444 nephrons, HFD HBW 18,757 ± 383 nephrons; P=0.252), with offspring of HFD NBW dams having, on average, 20% more nephrons per kidney than offspring of NFD NBW dams (15,005 ± 416 nephrons; P<0.0001). These data indicate that high fat feeding has a greater influence on offspring nephron endowment than maternal obesity.

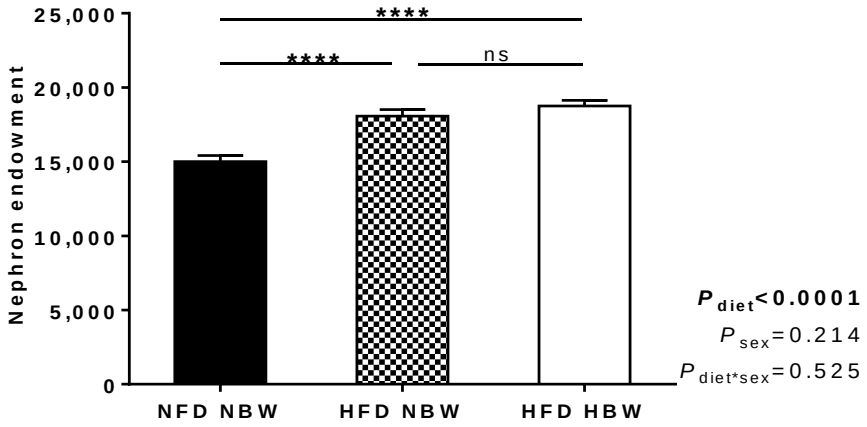


Figure 3.18 Nephron endowment at PN21 in offspring of high fat fed dams with normal body weight Nephron endowment in PN21 offspring of NFD NBW, HFD NBW and HFD HBW dams. Analysis by two-way ANOVA for maternal diet and offspring sex, weighted for litter using a mixed linear model. Values are mean ± SEM. Post-hoc analysis by Fisher's least significant difference. Values are mean ± SEM. *n*(NFD NBW)=12 kidneys from 6 litters, *n*(HFD NBW)=10 kidneys from 5 litters, *n*(HFD HBW)=14 kidneys from 8 litters. ****P<0.0001. NFD NBW=normal fat diet with normal body weight prior to pregnancy, HFD NBW=high fat diet with normal body weight prior to pregnancy, HFD HBW=high fat fed diet with high body weight prior to pregnancy.

3.5.2.5 Correlation analyses between maternal and offspring parameters and kidney development

Correlation and multiple regression analyses were used to examine relationships between nephron number and various maternal and offspring parameters at E18.5 and PN21.

3.5.2.5.1 Correlation and regression analyses between maternal and fetal parameters and kidney development at E18.5

Associations between various maternal and offspring variables with offspring kidney development at E18.5 are presented in **Table 3.8**. Offspring nephron number at E18.5 was strongly and positively associated with offspring body weight and kidney volume, and was also associated with maternal weight at conception and maternal pre-pregnancy glucose AUC. A multiple regression analysis was performed relating offspring nephron number to maternal weight at E0.5 and E18.5, maternal glucose AUC pre-pregnancy and fetal body weight. These variables explained 48% of the variation in offspring nephron number (R^2=0.483, P=0.0002). Fetal body weight (β=0.363, P=0.020) and maternal glucose prior to pregnancy (β=0.440, P=0.010) had significant positive regression weights, indicating that heavier offspring of glucose intolerant dams were expected to have a greater nephron number at E18.5. However, maternal weight did not contribute significantly to the multiple regression model.

Offspring body weight significantly associated with maternal weight at conception and at E18.5 but not maternal glucose AUC prior to pregnancy or in late gestation (**Table 3.8**). In a multiple regression model, maternal weight at E18.5 and maternal pre-pregnancy glucose AUC were found to moderately predict offspring body weight (R^2=0.290, *P*=0.003), with maternal weight contributing significantly to the model (β=0.523, *P*=0.002) above that of maternal glucose AUC (β=0.437, *P*=0.008).

Table 3.8 Spearman's rank coefficients for associations between nephron number at E18.5 and independent variables

		Offspring body weight	Offspring nephron number	Offspring kidney volume	Maternal weight E0.5	Maternal preg. weight gain	Maternal weight E18.5	Maternal glucose AUC pre-preg.
Offspring nephron number	**rho**	**0.518**						
	P	**0.0009**						
Offspring kidney volume	**rho**	**0.384**	**0.570**					
	P	**0.017**	**0.0002**					
Maternal body weight E0.5	**rho**	**0.329**	**0.354**	0.164				
	P	**0.043**	**0.029**	0.325				
Maternal preg. weight gain	**rho**	0.165	0.043	-0.022	0.113			
	P	0.621	0.822	0.897	0.498			
Maternal weight E18.5	**rho**	**0.327**	0.309	0.137	**0.757**	**0.707**		
	P	**0.045**	0.059	0.412	**<0.0001**	**<0.0001**		
Maternal glucose AUC pre-preg.	**rho**	0.251	**0.393**	**0.401**	-0.236	**-0.497**	**-0.446**	
	P	0.129	**0.015**	**0.013**	0.155	**0.002**	**0.005**	
Maternal glucose AUC E18.5	**rho**	-0.201	0.209	0.129	-0.133	**-0.363**	-0.252	0.202
	P	0.248	0.229	0.460	0.447	**0.032**	0.145	0.245

Spearman's rho (top value), *P* value (bottom value). *n*(NFD NGT)=21 kidneys from 7 litters, *n*(HFD IGT)=17 kidneys from 6 litters. Negative values indicate a negative association. Bold values are statistically significant.

The positive associations between maternal variables (maternal weight at E0.5 and maternal glucose AUC prior to pregnancy) and offspring nephron number are shown in **Figure 3.19A** and **Figure 3.19B**, respectively. The lack of an association between maternal glucose AUC in late pregnancy and offspring nephron number is shown in **Figure 3.19C**. The positive association between offspring body weight and nephron number at E18.5 is presented in **Figure 3.19D**.

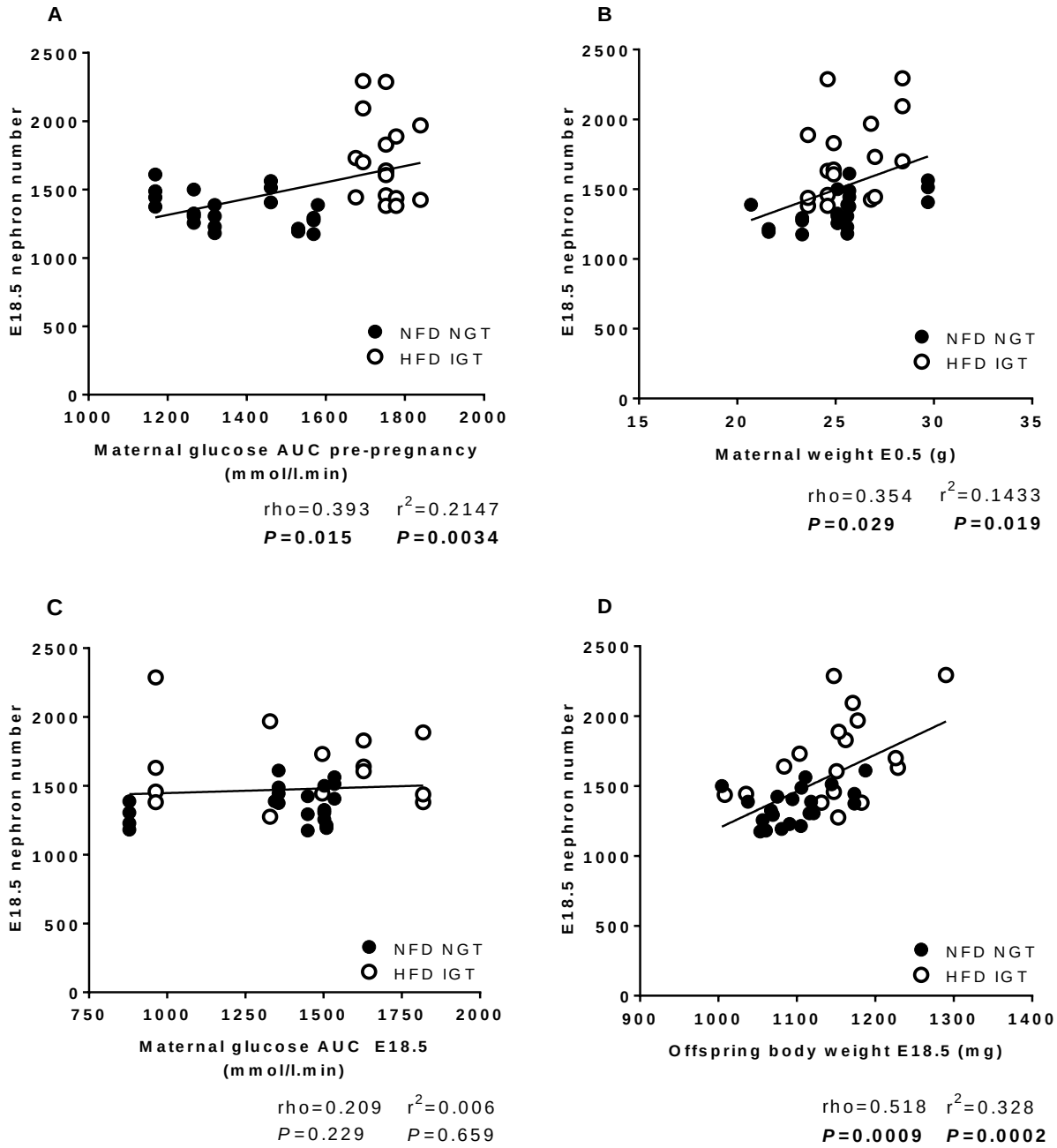


Figure 3.19 Associations between offspring nephron number at E18.5 and maternal glucose, maternal weight and offspring body weight

Plot of **A** maternal glucose AUC prior to pregnancy and **B** maternal weight at conception, **C** maternal glucose AUC at E18.5 and **D** offspring body weight at E18.5 against nephron number at E18.5 in offspring of NFD NGT dams and HFD IGT dams. *n*(NFD NGT)=21 kidneys from 7 litters, *n*(HFD IGT)=17 kidneys from 6 litters. Data analysis by Spearman's rho and linear regression. Solid line denotes line of best fit for combined data.

3.5.2.5.2 Correlation and regression analyses between maternal and offspring parameters and nephron endowment at PN21

The associations between various independent variables and offspring nephron endowment at PN21 are presented in **Table 3.9**. Unfortunately, as a number of dams that formed the PN21 cohort had missed vaginal seminal plugs, weight at conception was not sufficiently recorded and therefore gestational weight gain could not be included for analysis. Correlation and regression analyses include offspring of NFD NGT and HFD IGT dams, as well as offspring of HFD NGT dams.

Offspring nephron endowment was strongly and positively associated with kidney volume and maternal glucose AUC pre-pregnancy, and was also associated with maternal weight pre-pregnancy. A significant positive correlation was observed between offspring nephron endowment and body weight at PN21. A multiple regression analysis was performed relating offspring nephron endowment to offspring body weight, maternal glucose AUC and maternal weight. These variables explained 36% of the variation in offspring nephron endowment (R^2=0.361, *P*=0.001). Similar to findings at E18.5, offspring body weight (β=0.383, *P*=0.011) and maternal glucose AUC pre-pregnancy (β=0.365, *P*=0.021) contributed significantly to the model, indicating that heavier offspring were expected to have a greater nephron endowment, as were offspring of dams with greater glucose intolerance prior to pregnancy. Maternal body weight did not contribute to the multiple regression model (β=0.079, *P*=0.622).

Further analysis incorporating maternal diet (R^2=0.634, *P*<0.0001) revealed maternal diet to be the strongest predictor of offspring nephron endowment (β=0.696, *P*<0.0001), above that of offspring body weight *(*β=0.241, *P*=0.041). There was no significant contribution from maternal glucose or maternal body weight before pregnancy.

Offspring body weight at PN21 was not predicted by maternal diet, maternal glucose AUC or maternal body weight when analysed by multiple regression.

Table 3.9 Spearman's rank coefficients for associations between nephron endowment at PN21 and independent variables, including HFD NGT litters.

		Offspring body weight	Offspring nephron endowment	Offspring kidney volume	Maternal weight pre-pregnancy
Offspring nephron endowment	**rho**	**0.402**			
	P	**0.011**			
Offspring kidney volume	**rho**	**0.554**	**0.668**		
	P	**<0.0001**	**<0.0001**		
Maternal weight pre-pregnancy	**rho**	**0.366**	**0.414**	**0.356**	
	P	**0.022**	**0.009**	**0.026**	
Maternal glucose AUC pre-pregnancy	**rho**	0.162	**0.526**	0.183	**0.450**
	P	0.325	**0.0006**	0.265	**0.004**

Spearman's rho (top value), *P* value (bottom value). *n*(NFD NGT)=16 kidneys from 8 litters, *n*(HFD NGT)=12 kidneys from 7 litters, *n*(HFD IGT)=12 kidneys from 6 litters. Bold values are statistically significant.

The positive associations between offspring nephron endowment and maternal pre-pregnancy glucose AUC and maternal weight prior to conception are presented in **Figure 3.20A** and **Figure 3.20B**, respectively. Interestingly, nephron endowment appeared to reach an upper limit or threshold in offspring born to dams with very high glucose AUC values prior to pregnancy (**Figure 3.20A**). The association between offspring body weight and nephron endowment at PN21 is shown in **Figure 3.20C**.

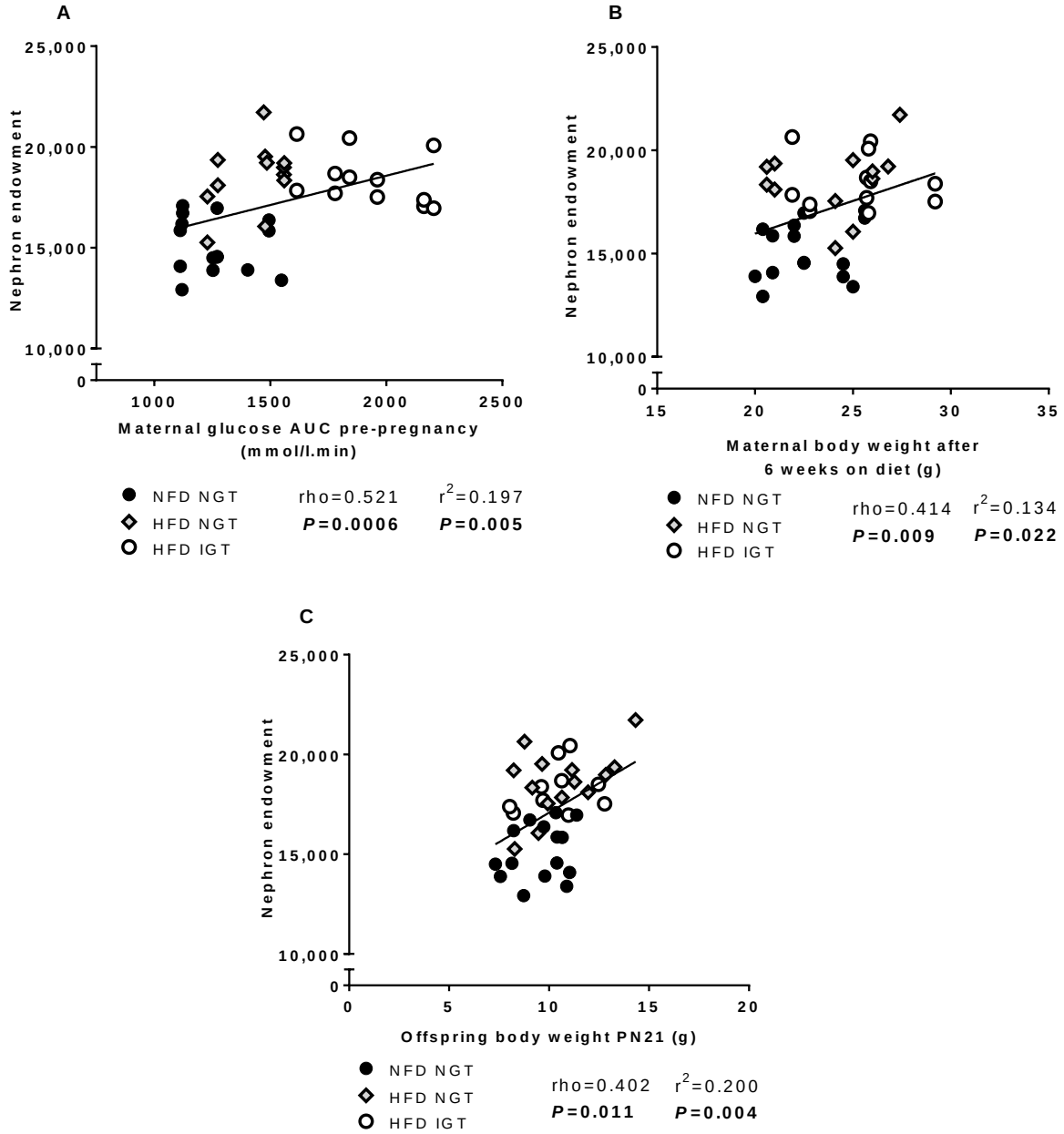


Figure 3.20 Associations between offspring nephron endowment at PN21 and maternal pre-pregnancy glucose AUC, maternal pre-pregnancy body weight and offspring body weight at PN21. Nephron endowment in PN21 offspring of NFD NGT, HFD NGT and HFD IGT dams plotted against **A** maternal pre-pregnancy glucose AUC, **B** maternal pre-pregnancy body weight and **C** offspring body weight at PN21. *n*(NFD NGT)=16 kidneys from 8 litters, *n*(HFD NGT)=12 kidneys from 7 litters, *n*(HFD IGT)=12 kidneys from 6 litters. NGT=normal fat diet with normal glucose tolerance, HFD IGT=high fat diet with impaired glucose tolerance, HFD NGT=high fat fed diet with normal glucose tolerance. Solid line denotes line of best fit for combined data.

3.5.3 Adult offspring metabolic function

A cohort of offspring from normal fat and high fat fed litters were weaned at PN21. Offspring were then fed standard chow and their metabolic function was assessed throughout adulthood.

3.5.3.1 Normal body weight and growth trajectories in adult offspring

Analysis by repeated measures ANOVA revealed no statistical difference in body weight trajectories amongst offspring of NFD NGT and HFD IGT dams between PN21 and 9 months of age (*P*=0.115, **Figure 3.21**). Although not of statistical significance, male offspring of HFD IGT dams tended to weigh less on average than offspring of NFD NGT dams from approximately 4 months of age, as illustrated in **Figure 3.21**. Weights of offspring at 4, 6 and 9 months of age are listed in **Table 3.10** and were not statistically different between groups. Male offspring were significantly heavier than female offspring at each time point (*P*<0.0001).

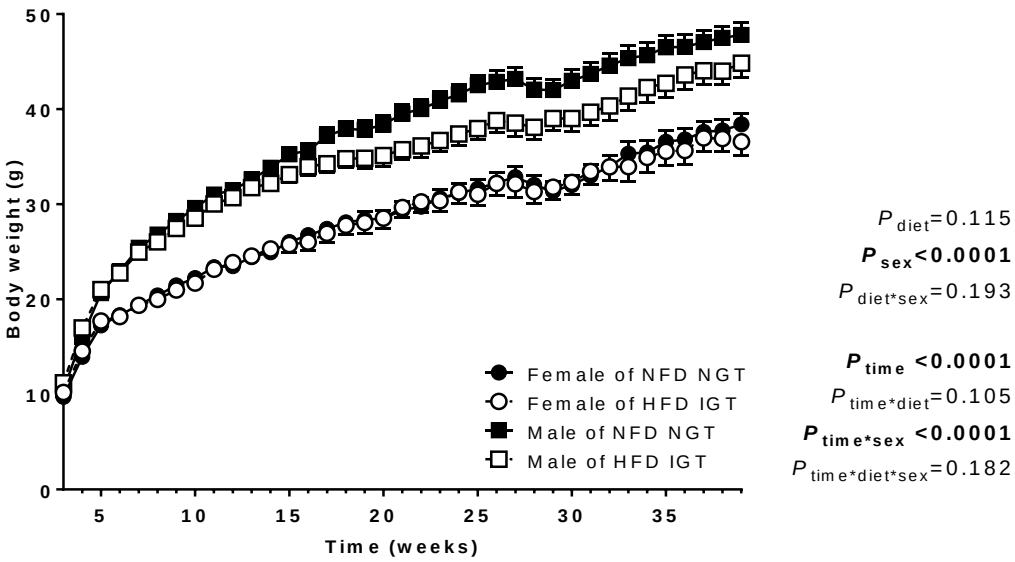


Figure 3.21 Offspring body weight trajectories after weaning

Body weight trajectories of offspring of NFD NGT and HFD IGT pregnancies between PN21 and 9 months of age. Data analysed by repeated measures ANOVA, mean weights per litter. Values are mean ± SEM. (*n*)NFD NGT female=20 offspring from 11 litters, (*n*)NFD NGT male=16 offspring from 9 litters, (*n*)HFD IGT female=11 offspring from 7 litters, (*n*)HFD IGT male=10 offspring from 7 litters.

Table 3.10 Offspring body weights at 4, 6 and 9 months of age

	Female of NFD NGT	Female of HFD IGT	Male of NFD NGT	Male of HFD IGT	P_{diet}	P_{sex}	$P_{diet*sex}$
4 month body weight, g	27.4 ± 0.9	27.1 ± 1.1	36.8 ± 0.9	34.4 ± 1.1	0.267	**<0.0001**	0.184
6 month body weight, g	32.1 ± 1.1	32.2 ± 1.5	42.0 ± 1.2	39.1 ± 1.6	0.423	**<0.0001**	0.148
9 month body weight, g	38.0 ± 1.1	36.7 ± 1.5	48.0 ± 1.3	44.8 ± 1.6	0.101	**<0.0001**	0.481

Analysis by two-way ANOVA incorporating maternal diet and sex, weighted for litter using mixed linear model. Values are mean ± SEM. *n*(NFD NGT female)=20 offspring from 11 litters, *n*(NFD NGT male)=16 offspring from 9 litters, *n*(HFD IGT female)=10 offspring from 7 litters, *n*(HFD IGT male)=10 offspring from 7 litters.

3.5.3.2 Normal body composition in adult offspring

DEXA scans were performed in offspring at 4 and 6 months of age and revealed no effect of maternal diet on offspring body composition (data not shown). However, at 9 months of age offspring of HFD IGT dams had a significantly lower bone area (*P*=0.039) and lean tissue mass (*P*=0.041) than offspring of NFD NGT dams (**Table 3.11**). There was no difference in other parameters of bone and body composition between the two groups. Male offspring had greater bone mineral content, bone area, lean tissue mass, total tissue mass and body fat percentage than female offspring at 9 months. Representative images of DEXA scans of 9 month offspring are presented in **Figure 3.22**.

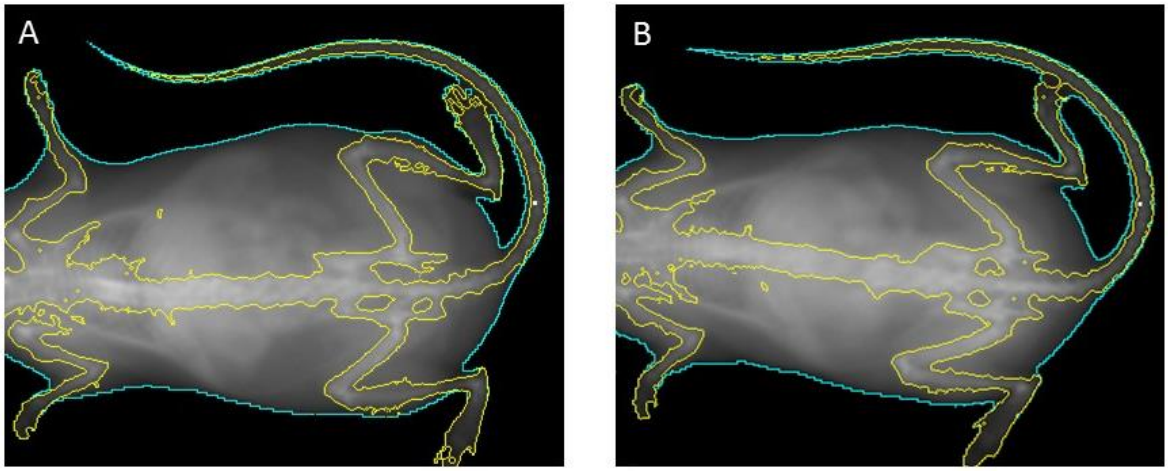


Figure 3.22 Representative DEXA scans of offspring at 9 months
Representative DEXA scans from 9 month old female offspring of **A** a NFD NGT **B** a HFD IGT dam.

Table 3.11 Offspring bone and body composition at 9 months

	Female of NFD NGT	Female of HFD IGT	Male of NFD NGT	Male of HFD IGT	P_{diet}	P_{sex}	$P_{diet*sex}$
Bone mineral density, g/cm²	0.052 ± 0.001	0.052 ± 0.001	0.052 ± 0.001	0.054 ± 0.001	0.519	0.450	0.319
Bone mineral content, g	0.505 ± 0.015	0.498 ± 0.019	0.582 ± 0.017	0.542 ± 0.021	0.199	**<0.0001**	0.352
Bone area, cm²	9.64 ± 0.23	9.53 ± 0.29	11.12 ± 0.25	10.05 ± 0.31	**0.039**	**0.001**	0.089
Lean tissue, g	20.95 ± 0.49	20.65 ± 0.63	29.23 ± 0.55	27.01 ± 0.67	**0.041**	**<0.0001**	0.113
Fat tissue, g	15.69 ± 1.00	14.39 ± 1.31	16.53 ± 1.13	15.48 ± 1.40	0.344	0.435	0.921
Total tissue, g	36.70 ± 1.15	34.99 ± 1.50	45.60 ± 1.29	42.60 ± 1.58	0.136	**<0.0001**	0.625
Percent body fat, %	41.90 ± 1.65	40.24 ± 2.17	36.01 ± 1.86	36.17 ± 2.33	0.714	**0.020**	0.656

Analysis by two-way ANOVA incorporating maternal diet and sex, weighted for litter using mixed linear model. Values are mean ± SEM. *n*(NFD NGT female)=20 offspring from 11 litters, *n*(NFD NGT male)=16 offspring from 11 litters, *n*(HFD IGT female)=12 offspring from 7 litters, *n*(HFD IGT male)=9 offspring from 7 litters.

3.5.3.3 Elevated fasting glucose but normal glucose tolerance in adult offspring of HFD IGT dams

There was no difference in offspring fasting glucose levels between maternal dietary groups at 4 months (NFD NGT 8.8 ± 0.3 mmol/l, HFD IGT 9.0 ± 0.4 mmol/l; *P*=0.737) or 6 months (NFD NGT 8.2 ± 0.3 mmol/l, HFD IGT 8.8 ± 0.4 mmol/l; *P*=0.186). However, fasting glucose levels were elevated in offspring of HFD IGT dams at 9 months (NFD NGT 8.0 ± 0.4 mmol/l, HFD IGT 9.6 ± 0.5 mmol/l; *P*=0.029). Fasting glucose was assessed in offspring across the postnatal period by repeated measures ANOVA **Figure 3.23**. Overall, there was a trend for offspring of HFD IGT dams to have elevated fasting glucose levels across adulthood (*P*=0.059). Overall, male offspring had greater fasting blood glucose concentrations than female offspring (*P*=0.039).

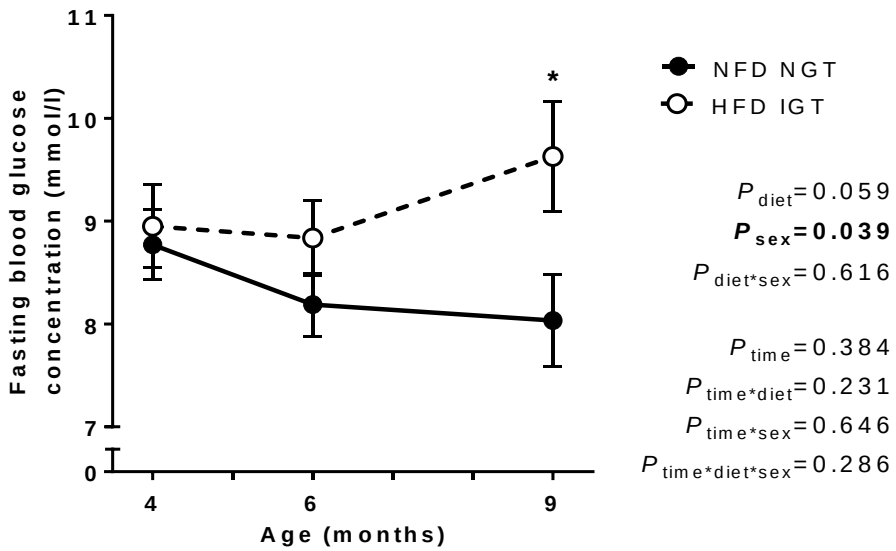


Figure 3.23 Fasting glucose in adult offspring
Fasting glucose values across the postnatal period in offspring of NFD NGT and HFD IGT dams. Analysis by repeated measures ANOVA incorporating maternal diet and offspring sex, with mean values of each litter analysed. Values are mean ± SEM. *n*(NFD NGT)=11 litters, *n*(HFD IGT male)=7 litters. **P*<0.05 refers to the difference between the two groups at that time point.

GTTs were performed in offspring at 4, 6 and 9 months of age. There was no statistical difference in glucose tolerance curves between offspring of NFD NGT and HFD IGT dams at 4 months (*P*=0.746; **Figure 3.24A**), 6 months (*P*=0.646; **Figure 3.24B**) or 9 months of age (*P*=0.159; **Figure 3.24C**). Male offspring had higher blood glucose profiles than female offspring at all time points (4 months: *P*<0.0001, 6 months: *P*<0.0001, 9 months: *P*=0.014). Glucose tolerance curves were also expressed as a glucose AUC value and analysed by repeated measures ANOVA. As shown in **Figure 3.24D**, there was no difference in glucose AUC between maternal dietary groups (*P*=0.922), although glucose AUC did increase in offspring of high fat dams from 6 to 9 months of age.

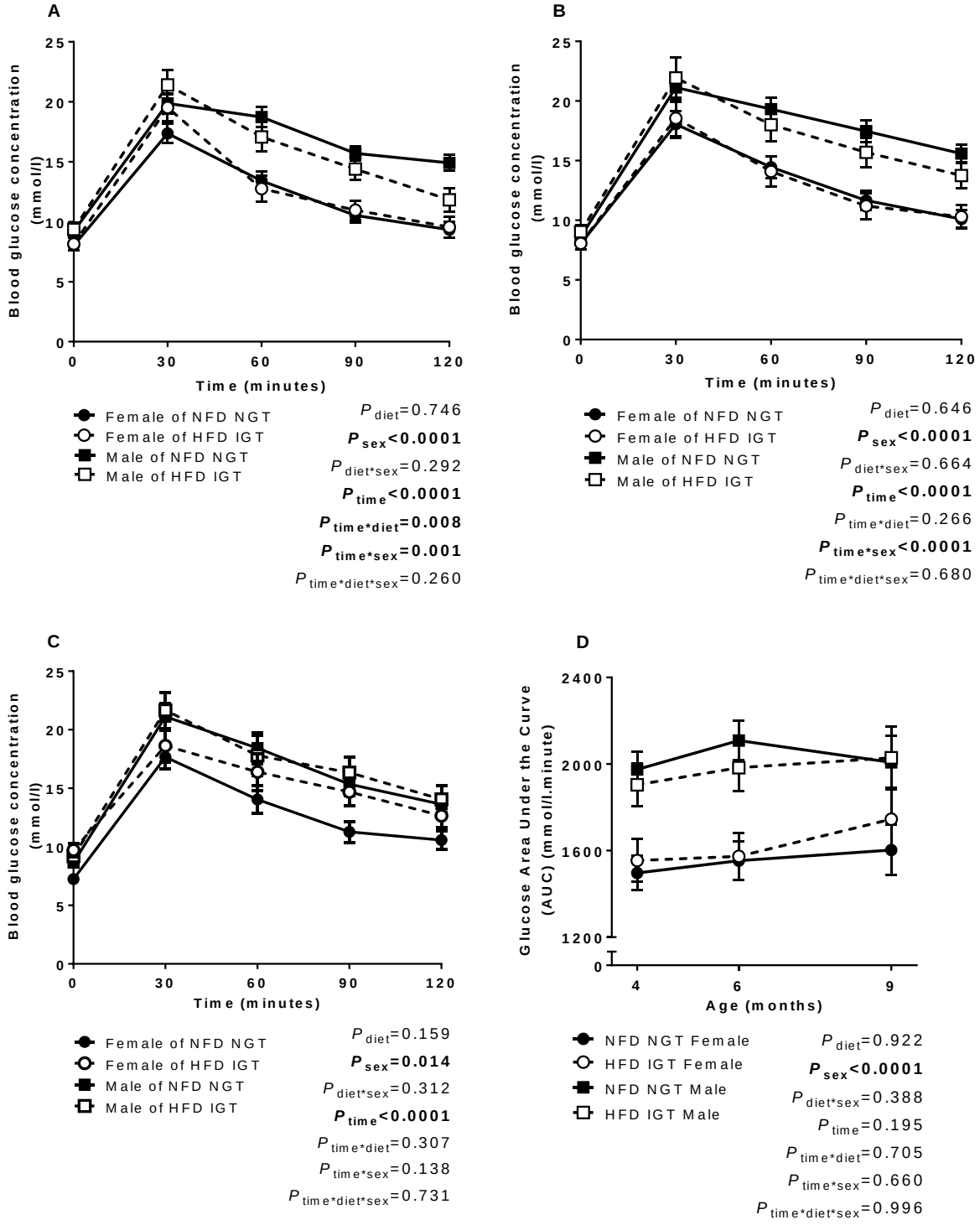


Figure 3.24 Glucose tolerance tests in adult offspring

GTTs at **A** 4 months, **B** 6 months and **C** 9 months in female and male offspring of NFD NGT and HFD IGT dams. Analysis by repeated measures ANOVA incorporating maternal diet and offspring sex. **D** Glucose AUC across the postnatal period in offspring of NFD NGT and HFD IGT dams. Analysis by repeated measures ANOVA incorporating maternal diet and offspring sex, with mean values of each litter analysed. Values are mean ± SEM. *n*(NFD NGT female)=21 offspring from 12 litters, *n*(NFD NGT male)=19 offspring from 12 litters, *n*(HFD IGT female)=11 offspring from 7 litters, *n*(HFD IGT male)=9 offspring from 7 litters.

3.5.4 Renal function

3.5.4.1 Normal renal clearance of FITC-sinistrin

At 6 months of age there was no difference in renal clearance of FITC-sinistrin (t half-life) between offspring of NFD NGT and HFD IGT dams (NFD NGT 13.80 ± 0.45 min, HFD IGT 13.99 ± 0.61 min; *P*=0.803; **Figure 3.25**). Male offspring had a significantly longer t half-life than female offspring (*P*<0.0001).

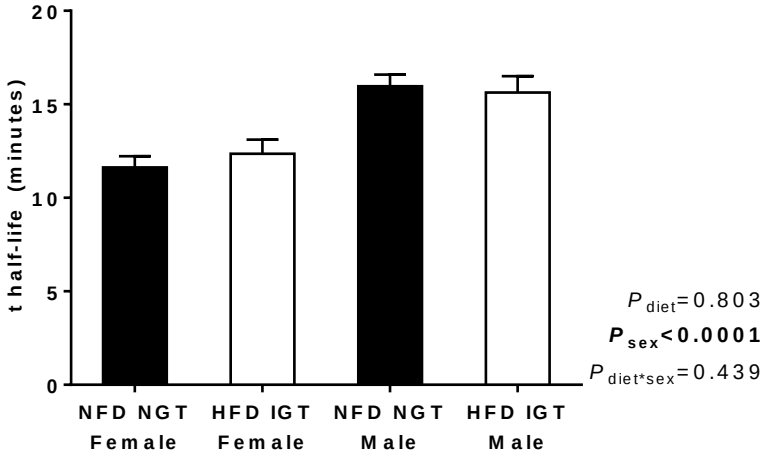


Figure 3.25 Offspring renal function at 6 months
FITC-sinistrin clearance (t half-life) in 6 month old offspring of NFD NGT and HFD IGT dams. Analysis by two-way ANOVA incorporating maternal diet and sex, weighted for litter using mixed linear model. Values are mean ± SEM. *n*(NFD NGT female)=18 offspring from 11 litters, *n*(NFD NGT male)=15 offspring from 11 litters, *n*(HFD IGT female)=11 offspring from 7 litters, *n*(HFD IGT male)=8 offspring from 7 litters.

At 9 months of age there was no difference in renal clearance of FITC-sinistrin (t half-life) between offspring of NFD NGT and HFD IGT dams (NFD NGT 13.49 ± 0.42 min, HFD IGT 13.90 ± 0.54 min; *P*=0.533; **Figure 3.26**). As seen at 6 months, t half-life was significantly greater in male offspring compared with female offspring (*P*<0.0001).

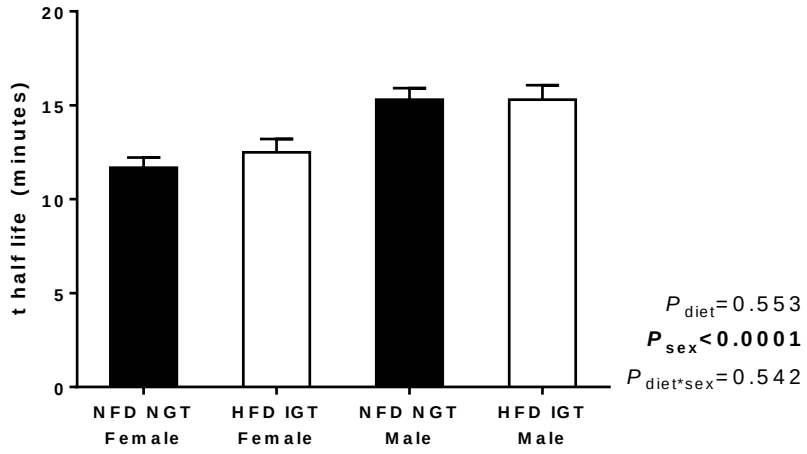


Figure 3.26 Offspring renal function at 9 months
FITC-sinistrin clearance (t half-life) in 9 month old offspring of NFD NGT and HFD IGT dams. Analysis by two-way ANOVA incorporating maternal diet and offspring sex, weighted for litter using mixed linear model. Values are mean ± SEM. *n*(NFD NGT female)=20 offspring from 11 litters, *n*(NFD NGT male)=16 offspring from 11 litters, *n*(HFD IGT female)=11 offspring from 7 litters, *n*(HFD IGT male)=9 offspring from 7 litters.

Renal function was also assessed in offspring across the postnatal period by repeated measures ANOVA. As shown in **Figure 3.27**, there was no difference in t half-life between 6 and 9 months of age in offspring of NFD NGT and HFD IGT fed dams (*P*=0.412).

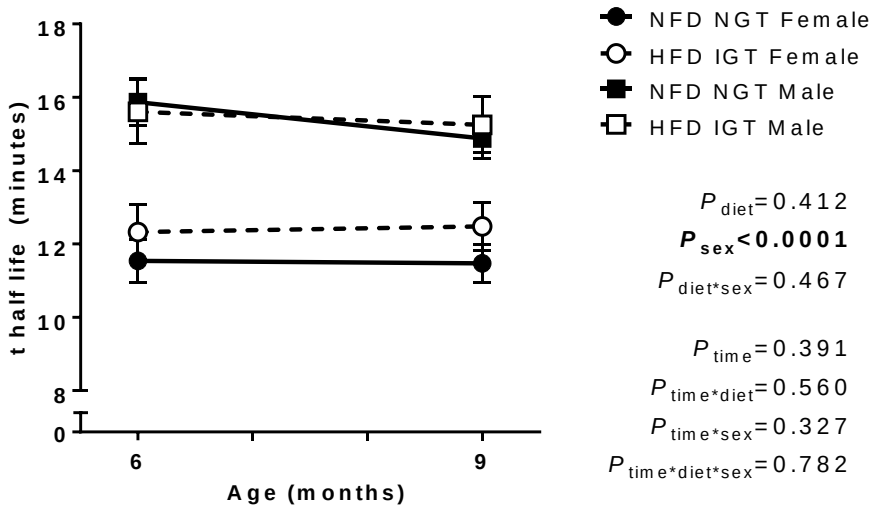


Figure 3.27 Offspring renal function over time
FITC-sinistrin clearance (t half-life) in 6 month and 9 month old offspring of NFD NGT and HFD IGT dams Analysis by repeated measures ANOVA incorporating maternal diet and offspring sex. Values are mean ± SEM. *n*(NFD NGT female)=20 offspring from 11 litters, *n*(NFD NGT male)=16 offspring from 11 litters, *n*(HFD IGT female)=11 offspring from 7 litters, *n*(HFD IGT male)=9 offspring from 7 litters.

3.5.4.2 Normal urine albumin excretion

As shown in **Figure 3.28**, there was no difference in the albumin: creatinine ratio in offspring of NFD NGT and HFD IGT dams (NFD NGT 6.62 ± 0.97 µg/mg, HFD IGT 6.82 ± 0.95 µg/mg, *P*=0.885).

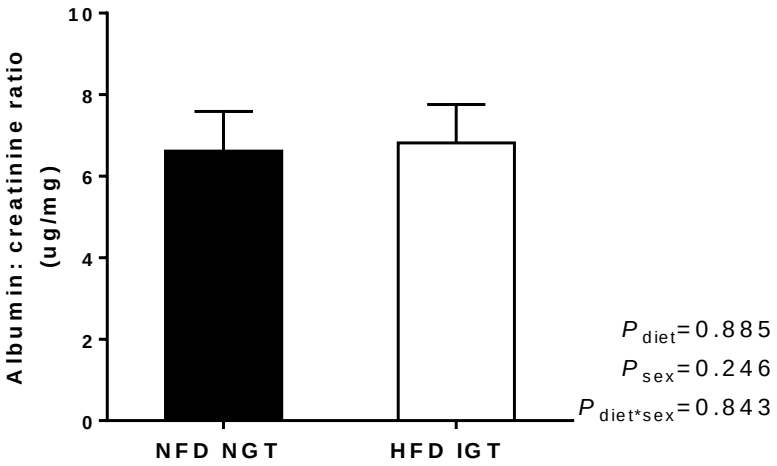


Figure 3.28 Offspring albumin: creatinine ratio at 9 months
Analysis by two-way ANOVA incorporating maternal diet and sex, weighted for litter using mixed linear model. Values are mean ± SEM. *n*(NFD NGT)=11 offspring from 8 litters, *n*(HFD IGT)=12 offspring from 8 litters.

3.5.5 Similar renal morphology

Kidney volume, total glomerular volume and total renal corpuscle volume in kidneys of 9 month offspring are presented in **Table 3.12**. The difference in kidney volume observed in PN21 littermates was not present at 9 months, with kidney volume similar in adult offspring of NFD NGT and HFD IGT pregnancies. Total glomerular volume and total renal corpuscle volume were also similar in the two groups. Male offspring had a larger kidney volume (*P*<0.0001), total glomerular volume (*P*=0.015) and total renal corpuscle volume (*P*=0.008) than female offspring at 9 months. However, when assessed relative to body weight, there was no difference in kidney volume, total glomerular volume or total renal corpuscle volume (**Table 3.12**).

Table 3.12 Kidney volume, total glomerular volume and total renal corpuscle volume of 9 month offspring

	Female of NFD NGT	Female of HFD IGT	Male of NFD NGT	Male of HFD IGT	P_{diet}	P_{sex}	$P_{diet*sex}$
Kidney volume, V_{kid}, mm^3	97.26 ± 6.50	103.62 ± 7.02	139.60 ± 7.02	142.50 ± 7.02	0.509	**<0.0001**	0.804
Total glomerular volume, $V_{glom,kid}$, mm^3	2.53 ± 0.21	2.44 ± 0.22	3.14 ± 0.23	3.06 ± 0.23	0.718	**0.015**	0.983
Total renal corpuscle volume, $V_{corp,kid}$, mm^3	3.58 ± 0.29	3.61 ± 0.32	4.73 ± 0.31	4.27 ± 0.32	0.528	**0.008**	0.401
Kidney volume: body weight, mm^3/g	2.70 ± 0.19	3.13 ± 0.21	3.04 ± 0.20	3.362 ± 0.21	0.072	0.160	0.788
Total glomerular volume: body weight, $V_{glom,kid}$, mm^3/g	0.071 ± 0.006	0.074 ± 0.006	0.068 ± 0.006	0.072 ± 0.006	0.564	0.743	0.918
Total renal corpuscle volume: body weight, $V_{corp,kid}$, mm^3/g	0.099 ± 0.007	0.108 ± 0.008	0.103 ± 0.008	0.100 ± 0.007	0.654	0.773	0.458

Analysis by two-way ANOVA incorporating maternal diet and sex, weighted for litter. Values are mean ± SEM. *n*(NFD NGT)=13 kidneys from 7 litters, *n*(HFD IGT)=12 kidneys from 6 litters.

3.5.6 Organ weights

Organ weights were assessed at 9 months of age.

3.5.6.1 Similar kidney weights

There was no difference in right kidney weight (NGT NFD 220.5 ± 6.6 mg, HFD IGT 229.0 ± 8.4 mg, *P*=0.434; **Figure 3.29A**) or left kidney weight (NGT NFD 207.4 ± 5.4 mg, HFD IGT 217.1 ± 7.0 mg, *P*=0.375; **Figure 3.29B**) between offspring of NFD NGT and HFD IGT dams. Similarly, there was no difference in combined kidney weight (NGT NFD 428.0 ± 11.7 mg, HFD IGT 446.5 ± 15.0 mg, *P*=0.343; **Figure 3.29C**) however there was a trend for offspring of HFD IGT dams to have a greater kidney weight to body weight ratio (NGT NFD 10.69 ± 0.37 mg/g, HFD IGT 11.86 ± 0.47 mg/g, *P*=0.051; **Figure 3.29D**). Male offspring had heavier kidneys than female offspring (*P*<0.0001).

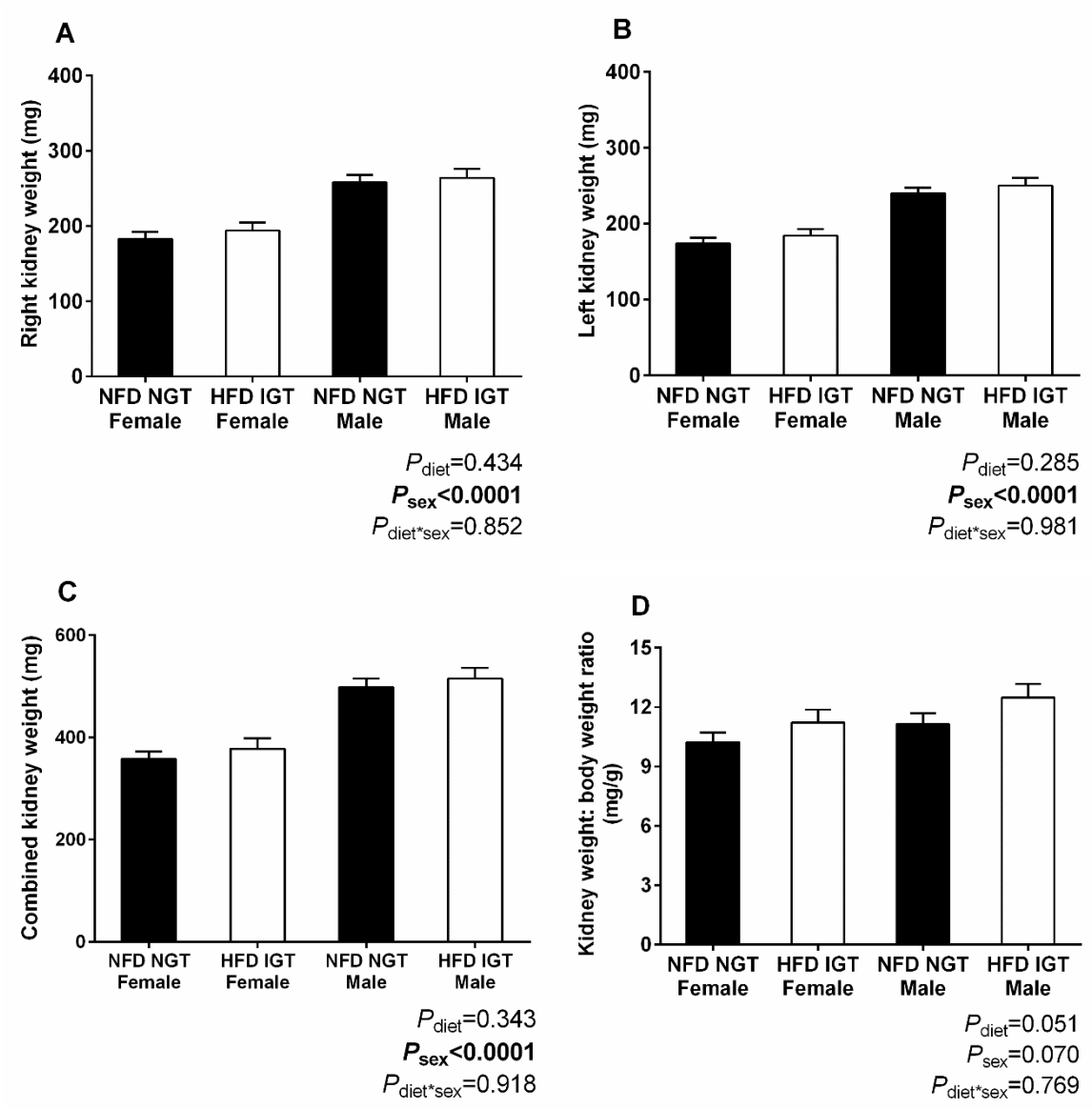


Figure 3.29 Offspring kidney weights at 9 months

A Right kidney weight, **B** left kidney weight, **C** combined kidney weight and **D** kidney weight to body weight ratio in offspring of NFD NGT and HFD IGT. Analysis by two-way ANOVA incorporating maternal diet and sex, weighted for litter using mixed linear model. Values are mean ± SEM. *n*(NFD NGT female)=20 offspring from 11 litters, *n*(NFD NGT male)=16 offspring from 11 litters, *n*(HFD IGT female)=11 offspring from 7 litters, *n*(HFD IGT male)=9 offspring from 7 litters.

3.5.6.2 Similar fat pad weights

Offspring of NFD NGT and HFD IGT dams had similar weights of mesenteric fat, renal fat and abdominal fat pads (**Table 3.13**). Male offspring had less renal fat than female offspring (*P*=0.006). There was no difference in combined fat mass between the two dietary groups (**Table 3.13**).

Table 3.13 Offspring fat pad mass at 9 months

	Female of NFD NGT	Female of HFD IGT	Male of NFD NGT	Male of HFD IGT	P_{diet}	P_{sex}	$P_{diet*sex}$
Mesenteric fat mass, mg	0.64 ± 0.07	0.63 ± 0.10	0.79 ± 0.08	0.78 ± 0.10	0.877	0.099	0.971
Renal fat mass, mg	1.15 ± 0.08	0.99 ± 0.12	0.75 ± 0.10	0.68 ± 0.12	0.302	**0.006**	0.671
Abdominal fat mass, mg	1.78 ± 0.13	1.68 ± 0.17	1.61 ± 0.15	1.87 ± 0.18	0.623	0.957	0.278
Combined fat mass, mg	3.58 ± 0.26	3.29 ± 0.35	3.18 ± 0.30	3.33 ± 0.37	0.826	0.574	0.511

Analysis by two-way ANOVA incorporating maternal diet and sex, weighted for litter using mixed linear model. Values are mean ± SEM. *n*(NFD NGT female)=20 offspring from 11 litters, *n*(NFD NGT male)=16 offspring from 11 litters, *n*(HFD IGT female)=11 offspring from 7 litters, *n*(HFD IGT male)=9 offspring from 7 litters.

3.6 Discussion

In the present chapter, offspring of dams with diet-induced obesity and glucose intolerance were studied to investigate the effect of pre-gestational Type 2 diabetes on kidney development and adult renal health. Offspring were found to be macrosomic prior to birth with normal body weight at weaning. Exposure to a maternal high fat diet did not alter ureteric tree development or nephrogenesis in offspring at E15.5. Surprisingly however, by E18.5 the number of nephrons was significantly higher in offspring of high fat fed dams. This increase in nephron number was established at a relatively early time point in nephrogenesis and was maintained at PN21 following the completion of nephrogenesis. Offspring were also examined in adulthood to assess the long-term consequences of pre-gestational Type 2 diabetes on metabolic health and renal function. Offspring had normal body weight, glucose tolerance and body composition during the 9 month study period and renal function and renal morphology were also normal at 9 months of age. These results and their implications are discussed below.

3.6.1 Embryonic and early postnatal growth

Neonatal macrosomia is a common feature of human pregnancies complicated by maternal diabetes (HAPO Study Cooperative Research Group, 2008, Reece, 2010). As glucose but not insulin readily crosses the placenta, increases in fetal glucose levels stimulate the fetal pancreas to overproduce insulin, the main fetal growth factor. Consequently, excess insulin increases fetal glucose utilisation and its conversion to FFAs, thus promoting fetal adiposity and overgrowth as first postulated by Pedersen (1954). As insulin resistance and obesity are associated with an increase in circulating FFAs and triglycerides in addition to glucose, maternal lipids may also contribute to fetal overgrowth and adiposity (Freinkel, 1980). Maternal triglycerides, once hydrolysed to FFAs, are able to cross the placenta and incorporate into fetal lipids (Knopp et al., 1985). Levels of maternal triglycerides and FFAs correlate with birth weight, neonatal abdominal circumference and fat mass (Kitajima et al., 2001, Schaefer-Graf et al., 2008, Scifres et al., 2011), highlighting how fetal growth is a function of numerous nutritional factors (Catalano and Hauguel-De Mouzon, 2011).

Rodent studies of pre-gestational high fat feeding have reported offspring macrosomia (Samuelsson et al., 2008, Jones et al., 2009, Ashino et al., 2012, Li et al., 2013), although increases in fetal and neonatal weights are not universally reported (Holemans et al., 2004, Taylor et al., 2005, Srinivasan et al., 2006a, Ferezou-Viala et al., 2007, Nivoit et al., 2009). This may be related to differences in dietary lipid composition, maternal and fetal lipid, glucose and insulin profiles and the degree of fetal pancreatic involvement. It is also important to note that body fat content at birth differs between species, with rodents having a lower body fat percentage (1-3%) than humans (12-15%) (Widdowson,

1950). Fetal pancreatic development also differs in humans and rodents, as the human fetal pancreas begins to secret insulin from mid gestation whereas nutrient stimulated insulin secretion does not occur until very late in gestation in mice and rats (Mendonca et al., 1998, Pan and Wright, 2011). Lower fat content at birth and delayed functional maturation of the fetal pancreas may also contribute to discrepancies in offspring macrosomia in rodent models of high fat feeding.

In the present study, offspring of high fat fed dams were macrosomic at E18.5 with no difference in crown-rump length, head diameter or ponderal index, indicating that offspring underwent symmetric growth. Previous rodent models of macrosomia report fetal hyperinsulinaemia (Lawrence et al., 1989, Srinivasan et al., 2006a) yet fetal plasma insulin levels were not significantly elevated in the present study. The increase in fetal weight was observed despite normal maternal glucose tolerance and maternal body weight in late gestation, and was best predicted by maternal weight prior to pregnancy but not maternal weight at E18.5, maternal glucose AUC prior to pregnancy or maternal glucose AUC at E18.5. There is some evidence indicating that exposures in early pregnancy may cause changes in early placental growth, function and gene expression, which may determine how the placenta will respond to exposures in late pregnancy (Ericsson et al., 2007, Lewis et al., 2013, Diouf et al., 2014). In the present study, maternal obesity in early pregnancy may have altered early placental development and subsequent placental nutrient transfer, leading to fetal overgrowth independent of maternal weight in late pregnancy.

Maternal hyperlipidaemia may have also contributed to the observed macrosomia (Schaefer-Graf et al., 2008). Transplacental transfer of fatty acids is driven by the maternal to fetal concentration gradient as well as the expression of lipoprotein receptors, lipase activity and FABPs in the placenta (Haggarty, 2010). While there is a paucity of studies examining the regulation of fetal overgrowth in maternal obesity, enhanced expression of placental FATs and lipoprotein lipase have been reported in conjunction with fetal lipid accumulation and fetal overgrowth in experimental models of maternal obesity (Zhu et al., 2010, Mazzucco et al., 2013). Further studies are required to assess whether the transfer of lipids in placentae of high fat fed dams was similarly upregulated at E18.5, and to assess early and late placental morphology in high fat fed dams.

There was no difference in body weight in the early postnatal period between offspring of normal fat and high fat fed dams. Pups were not weighed until PN3 to reduce the rate of cannibalism and consequently offspring body weights between E18.5 and PN3 are unknown. Increased pup weights have been reported in offspring of high fat fed dams at weaning (Samuelsson et al., 2008, Nivoit et al., 2009, Ashino et al., 2012), yet glucose profiles and body weights of dams in the postnatal period are poorly reported. In the present study high fat fed dams had normal glucose tolerance and body weight

in late gestation and during lactation. The increased body weight observed in offspring of high fat fed dams prior to birth but not in the early postnatal period may be due to differential nutrient transfer across the placenta versus lactation.

3.6.2 Kidney development

In the present study it was hypothesised that exposure to maternal glucose intolerance would be detrimental to offspring kidney development. This hypothesis was based on previous reports of reduced nephron number in offspring of STZ-treated hyperglycaemic dams. Commonly, STZ-induced hyperglycaemia is associated with fetal growth restriction (Amri et al., 1999, Tran et al., 2008, Hokke et al., 2013) which may have its own confounding effects on kidney development given the positive association between birth weight and nephron number (Hughson et al., 2003). Reduced nephron endowment has, however, been reported in normal body weight offspring exposed to maternal hyperglycaemia induced by STZ treatment and continuous glucose infusion (Amri et al., 1999). In the present study, it was hypothesised that offspring of dams with diet-induced obesity and glucose intolerance would be macrosomic and that, despite fetal overgrowth, kidney development would be negatively affected given the detrimental effects of hyperglycaemia on nephron formation (Kanwar et al., 2005, Tran et al., 2008, Chen et al., 2011). However, the present findings, as discussed below, do not support this hypothesis. A possible explanation for this is that offspring of high fat fed dams were not exposed to maternal glucose intolerance during kidney development.

Pre-gestational Type 2 diabetes induced by high fat feeding had no effect on kidney development in offspring at E15.5. Ureteric tip number, ureteric tree length and ureteric tree volume, as well as branch generation number and tree segment lengths, were similar between offspring of normal fat and high fat fed dams. Offspring nephron number was also similar in the two groups. The lack of an effect on kidney development at E15.5 is likely related to the maternal phenotype at this time. While high fat fed dams were heavier than normal fat fed dams at E15.5, maternal glucose tolerance was similar between the two groups at this time. Unfortunately, GTTs were not performed prior to E15.5 and therefore the duration of glucose intolerance in pregnancy in high fat fed dams is unknown. This is an important consideration given that murine metanephric development commences at E10.5. If maternal glucose levels were comparable in high fat and normal fat fed dams in the days leading up to E15.5, it follows that ureteric tree development and early nephron formation would not be affected.

A dramatically different phenotype was observed in offspring of high fat fed dams at E18.5. Just prior to birth, offspring of high fat dams had more nephrons and larger kidney volumes than offspring of normal fat fed dams. This increase in nephron number and kidney size was accompanied by offspring macrosomia at E18.5, although elevations in nephron number and kidney volume were still present

following adjustment for body weight. Regression analyses found E18.5 nephron number was best predicted by fetal body weight and maternal glucose intolerance prior to pregnancy but not maternal weight.

Final nephron number was also greater in high fat fed offspring at weaning. The proportionate increase in nephron number observed at E18.5 (25%) and PN21 (20%) indicates that no additional acceleration in nephron formation occurred after birth. As seen at E18.5, nephron endowment at PN21 was best predicted by maternal glucose intolerance prior to pregnancy and offspring body weight. After adjusting for maternal diet, there was no contribution of maternal glucose tolerance or maternal body weight on offspring nephron endowment. This may indicate that the increase in nephron endowment in offspring of high fat fed dams is related to certain dietary constituents that cross to the offspring and promote nephrogenesis, rather than maternal diet-induced obesity or glucose intolerance. This is supported by the findings that nephron endowment was increased in offspring of high fat fed dams regardless of the degree of glucose intolerance or obesity prior to pregnancy. The factors that may have promoted nephrogenesis in high fat fed offspring in late gestation are considered below.

3.6.3 Factors promoting nephrogenesis

Experimental studies have identified a range of environmental perturbations that can detrimentally affect kidney development and reduce offspring nephron endowment. These perturbations include, but are not limited to: maternal food restriction, maternal low-protein diet, exposure to glucocorticoids, alcohol and antibiotics, vitamin A deficiency, placental insufficiency and maternal hyperglycaemia (Hoy et al., 2010, Luyckx and Brenner, 2010, Walker and Bertram, 2011). Conversely, few studies have reported increased nephron endowment due to an altered maternal environment. However, increased nephron endowment has been reported in offspring exposed to maternal vitamin D deficiency (Maka et al., 2008) and maternal water restriction (Mansano et al., 2007) as well as in offspring of dams administered retinoic acid (Lelievre-Pegorier et al., 1998). Of relevance to the present study is the increase in offspring nephron endowment due to postnatal hypernutrition reported by Boubred et al. (2007). Using a rat model of early postnatal overnutrition induced by the reduction of litter size at PN3 (reduced to 3 pups per litter), Boubred et al. (2007) observed a 35-40% increase in pup bodyweight at PN7 and PN21. This acceleration in pup growth in the early suckling period, during a key window of profuse postnatal nephrogenesis, was associated with a 20% increase in nephron endowment. While Boubred et al. (2007) did not examine the mechanisms promoting postnatal nephrogenesis, they postulated that an increase in the availability of energy and nutritional substrates and growth factors within the kidney likely contributed to the increase in nephron endowment. Similar mechanisms may also have played a part in the present study.

In order to isolate the contribution of maternal high fat feeding *per se* on offspring kidney development, nephron endowment was determined in offspring of high fat fed dams who displayed comparable glucose AUC values prior to pregnancy to that of normal fat fed dams. PN21 offspring of high fat dams with normal glucose tolerance were found to have a greater nephron endowment than offspring of normal fat dams. Moreover, this increase was equivalent to that observed in offspring of high fat dams with impaired glucose tolerance. Nephron endowment was also elevated in PN21 offspring of high fat fed dams regardless of the degree of maternal obesity prior to pregnancy. These analyses highlight that a maternal high fat diet augments offspring nephrogenesis despite having variable effects on maternal phenotype prior to pregnancy, specifically glucose handling and obesity.

It is important to mention that the stimulatory effects of maternal high fat feeding on offspring nephrogenesis were only observed at E18.5 and in the early postnatal period. Despite a 25% increase in nephron number at E18.5 compared with offspring of normal fat feeding, maternal high fat feeding did not enhance ureteric branching morphogenesis or nephron number just 3 days prior at E15.5. Unfortunately, due to the technical limitations of Tree Surveyor software it was not possible to assess ureteric tree development by OPT at E18.5. While the rate of ureteric branching morphogenesis is known to slow in late gestation (Cebrián et al., 2004), nephron formation is initiated adjacent to ureteric tips and it is possible that ureteric tree development was accelerated in conjunction with nephron formation after E15.5. Numbers of cap mesenchyme cells, the progenitor cells that surround ureteric buds from which nephrons form (Kopan et al., 2014), may also be increased in offspring of high fat fed dams at E15.5 given the later increase in nephron number. Given the comparable increase in nephron number at E18.5 and PN21, it is unlikely that the length of nephrogenesis was prolonged in offspring of high fat fed dams. The acceleration in nephron number may be possibly attributed to an increased formation of nephron arcades. Further studies assessing and quantifying cap mesenchyme cell populations and nephron arcade formation are required to determine if this is the case.

The contrasting nephron number phenotypes observed at E15.5 and E18.5 may also be due to placental maturity and temporal changes in the transfer of nutritional substrates from the maternal to fetal circulation. Previously, Lin et al. (2012) found differential mRNA expression of placental nutrient transporters in a maternal high fat environment. Placental GLUT3 and cationic amino acid transporter were upregulated in high fat fed rats with fetal overgrowth at E17.5, but had normal expression levels at E13.5. Unfortunately, additional studies assessing nutrient transfer profiles between mid and late gestation in high fat fed dams are sparse. Single time point studies show upregulated expression of other placental nutrient transporters including GLUT1, sodium-coupled neutral amino acid transporter (SNAT) 2 and lipoprotein lipase in late gestation high fat fed dams with fetal overgrowth (Jones et al., 2009, Mazzucco et al., 2013). This was associated with an accumulation of nutrients in the fetal

compartment, with Jones et al. (2009) showing enhanced unidirectional clearance of glucose and amino acids from the maternal to fetal circulation, and Mazzucco et al. (2013) reporting increased fetal hyperglycaemia and hypertriglyceridaemia in high fat fed dams. It should be noted however, that the expression profiles of specific placental transporters in animal models of high fat feeding are inconsistent (Lin et al., 2012, Gaccioli et al., 2013b). Furthermore, whether the upregulation of placental nutrient transporters occurs in response to fetal overgrowth or whether the temporal expression of nutrient transporters is altered in response to a maternal high fat diet *per se* is yet to be determined. Nonetheless, numerous studies show an increase in transplacental transport of glucose, amino acids and triglycerides in late gestation high fat fed dams. This in turn provides more energy and metabolic substrates to the fetus to promote growth. If extrapolated to the present study, increased placental transfer of nutritional substrates in high fat fed dams may have contributed to offspring macrosomia and augmented kidney growth and nephrogenesis in late gestation.

3.6.4 Adult metabolic health

Offspring of normal and high fat fed dams were studied until 9 months of age. There was no effect of pre-gestational Type 2 diabetes induced by maternal high fat feeding on offspring body weight in adulthood. Previous studies of maternal high fat feeding report a 10-20% greater offspring body weight by approximately 3 months of age compared with offspring of dams fed a normal fat diet (Samuelsson et al., 2008, Ashino et al., 2012, Masuyama and Hiramatsu, 2014). These findings were observed in offspring of overweight dams with insulin and leptin resistance. Conversely, studies of maternal overnutrition by Jackson et al. (2012) and Ferezou-Viala et al. (2007) report similar offspring weight in adulthood yet provide no data on maternal plasma analytes. Considerable sexual dimorphism is also reported in studies of maternal high fat feeding with greater body weights observed in only male (Nivoit et al., 2009) or female (Khan et al., 2003, Elahi et al., 2009) littermates compared with controls.

Interestingly, most experimental studies have reported an association between neonatal macrosomia and later weight gain. With the exception of Nivoit et al. (2009) who reported reduced neonatal pup weight (owing to an increase in litter size), offspring of high fat fed dams with neonatal macrosomia exhibit increased weight in adulthood (Samuelsson et al., 2008, Ashino et al., 2012, Masuyama and Hiramatsu, 2014) whereas offspring with normal neonatal body weight do not exhibit overweight in adulthood (Ferezou-Viala et al., 2007, Jackson et al., 2012). Given that offspring of high fat dams in the present study were macrosomic at E18.5, and that high fat fed dams were overweight, hyperinsulinaemic and had a greater energy intake in pregnancy, the findings of the present study do not reflect that of the literature.

Alongside increases in body weight, adult offspring exposed to maternal overnutrition typically display increased adiposity. In both mice and rats, greater fat pad mass and percentage body fat have frequently been reported in overweight offspring of high fat fed dams (Samuelsson et al., 2008, Elahi et al., 2009, Nivoit et al., 2009, Ashino et al., 2012, Masuyama and Hiramatsu, 2014). Raised serum triglycerides levels and liver triglyceride content, as well as adipocyte hypertrophy and liver lipid vacuoles were also reported in these studies (Samuelsson et al., 2008, Elahi et al., 2009, Ashino et al., 2012, Masuyama and Hiramatsu, 2014). In the present study, individial and combined fat pad masses were similar in offspring of normal and high fat dams at 9 months of age. Likewise, DEXA scans performed at 4, 6 and 9 months showed no difference in percentage body fat between the two groups. These findings are not surprising considering body weights were comparable between the two dietary groups in the postnatal period, although increased adiposity has been observed in normal weight offspring exposed to maternal high fat diet during gestation and lactation (Khan et al., 2005).

In the present study, offspring born to high fat fed dams displayed fasting hyperglycaemia at 9 months of age, but not at 4 or 6 months. The increase in fasting blood glucose concentration over time may suggest an inability for offspring of high fat fed dams to maintain glucose homeostasis with advancing age, as shown previously (Taylor et al., 2005, Srinivasan et al., 2006b, Samuelsson et al., 2008). Fasting hyperglycaemia has been reported in offspring of high fat fed dams as early as 3 months of age (Masuyama and Hiramatsu, 2014), although most studies report normal fasting glucose levels (Ferezou-Viala et al., 2007, Nivoit et al., 2009, Ashino et al., 2012, Jackson et al., 2012). The variable effect on fasting glycaemia may be related to offspring age and whether associated overweight and insulin resistance were present. Nonetheless, maternal overnutrition does seem to have an inconsistent effect on fasting glucose levels in adult offspring born to dams fed a high fat diet. Again, the timing and duration of the high fat diet, the species and strain studied, and the source of dietary fat may all influence outcomes.

Reports on the effect of maternal high fat feeding on offspring glucose tolerance also vary. However, previous reports agree that there is a clear correlation between fasting glucose values and glucose tolerance whereby adult offspring with fasting hyperglycaemia display impaired glucose tolerance and raised glucose AUC values in a GTT (Srinivasan et al., 2006b, Masuyama and Hiramatsu, 2014), whereas adult offspring with normal fasting glucose values exhibit normal glucose tolerance (Nivoit et al., 2009, Ashino et al., 2012, Jackson et al., 2012). In consideration of this, it is interesting that in the present study offspring of high fat dams had normal glucose tolerance despite fasting hyperglycaemia at 9 months of age. It is plausible that glucose intolerance may have presented in older offspring and further experiments to assess glucose handling in offspring at 12 months of age or older are required.

In the present study, offspring of high fat fed dams were hyperglycaemic despite normal body weight and body composition which may suggest a primary pancreatic issue or skeletal insulin resistance not mediated by obesity. Additional experiments should assess adult offspring of high fat fed dams for insulin resistance and pancreatic dysfunction. Insulin resistance has been frequently reported in adult offspring of high fat fed dams with studies reporting an increase in fasting plasma insulin and glucose stimulated insulin secretion, reduced insulin sensitivity and insulin resistance (Taylor et al., 2005, Srinivasan et al., 2006b, Samuelsson et al., 2008, Nivoit et al., 2009, Ashino et al., 2012, Jackson et al., 2012, Masuyama and Hiramatsu, 2014), which in a chronic situation may result in frank diabetes and β-cell exhaustion (Taylor et al., 2005, Samuelsson et al., 2008).

3.6.5 Adult renal health

In the present study, pre-gestational Type 2 diabetes and maternal high fat feeding did not affect offspring renal function or renal morphology. Offspring of high fat fed dams displayed normal renal clearance of FITC-sinistrin at 6 and 9 months of age, with no evidence of albuminuria or glomerular hypertrophy at 9 months of age compared with offspring of normal fat fed dams.

Studies assessing developmental programming of renal function and structure in maternal overnutrition are limited. In a study by Armitage et al. (2005), offspring of rats fed a high fat diet for 10 days prior to mating, throughout gestation and lactation were hypertensive at 6 months of age (Khan et al., 2003), yet with normal nephron number and glomerular volume. Interestingly, increased blood pressure was confined to female offspring. In a study by Jackson et al. (2012), male offspring of rats fed a high fat, high fructose diet for 6 weeks prior to pregnancy, throughout gestation and lactation, were found to have normal blood pressure and GFR yet increased urinary albumin excretion and renal pathology including glomerulosclerosis and tubulointerstitial fibrosis by 4 months of age. Unfortunately, GFR and albuminuria were not reported by Armitage et al. (2005) and nephron number and glomerular volume were not assessed by Jackson et al. (2012). Without full analysis of renal function and renal morphology it is difficult to fully elucidate the effect maternal overnutrition has on the adult kidney.

Few studies have assessed the long-term consequences of elevated nephron endowment. Accelerated nephrogenesis has been reported in mice transgenic for members of the TGF-β superfamily including TGF-β2, betaglycan and type II Activin receptor (Maeshima et al., 2000, Sims-Lucas et al., 2008, Walker et al., 2011). Particular attention has been given to TGF-β2, a signalling molecule that negatively regulates ureteric branching morphogenesis. Heterozygous null TGF-β2 mice show a 30-60% increase in nephron endowment alongside reduced mean glomerular volume (Sims-Lucas et al., 2008, Walker et al., 2012). These mice also displayed normal urine osmolality, albumin excretion, creatinine

clearance and blood pressure at 4-6 months of age (Walker et al., 2012). Based on these studies, elevated nephron endowment does not seem to influence renal function or blood pressure when induced by genetic dysregulation of nephrogenesis.

Conversely, renal function and blood pressure do seem to be influenced by elevated nephron endowment induced by a perturbed environmental stimulus. As mentioned previously, Boubred et al. (2007) reported a 20% increase in nephron endowment in rat pups exposed to early postnatal hypernutrition. Offspring with elevated nephron number displayed raised systolic blood pressure as early as 2 and 4 months of age. At 12 months of age overfed male offspring were hypertensive with increased protein excretion rate and glomerulosclerosis, features that were not observed in aged overfed females despite a comparable increase in nephron endowment. Interestingly, there was no effect of postnatal hypernutrition on GFR, despite overfed male offspring exhibiting relative glomerular hypertrophy. The reasons for sexual dimorphism in this study are unclear, but may be related to overfed female offspring exhibiting lower mean glomerular volumes. This suggests that their global glomerular filtration surface area was unaffected, possibly protecting them from albuminuria and hypertension. Studies performed in heterozygous null TGF-β2 mice support this, as an increase in nephron number accompanied by a decrease in mean glomerular volume was associated with normal renal function and blood pressure (Sims-Lucas et al., 2008, Walker et al., 2012).

The protective effect a high nephron endowment may exert on renal function and glomerular morphology has been shown in heterozygous TGF-β2 mice following a chronic insult. Chronic exposure to a high salt diet raised mean arterial pressure in wild type mice, but not heterozygous TGF-β2 mice. The blunted arterial pressure response in mice with a high nephron endowment was likely due to a lower single nephron GFR, protecting mice from hyperfiltration-induced injury. The protective effect of a high nephron endowment was not observed in male rat offspring exposed to early postnatal hypernutrition (Boubred et al., 2007). This may be related to glomerular hypertrophy, but may also be due to the long-term effects that overnutrition during development has on metabolic health and function. In the study by Boubred et al. (2007), offspring were slightly, but not significantly heavier in adulthood despite considerable overgrowth in the early postnatal period. Adiposity and glucose tolerance were not measured, yet it is possible that postnatal overnutrition may have programmed features of metabolic syndrome in addition to hypertension that, in turn, may be detrimental to renal function and structure. Metabolic syndrome is a known risk factor for CKD and microalbuminuria (Chen et al., 2004). Obesity, glucose intolerance and insulin resistance are also positively and independently associated with glomerular hypertrophy, hyperfiltration and glomerulosclerosis (Griffin et al., 2008, Tobar et al., 2013). The mechanisms by which obesity, glucose intolerance and high triglyceride levels damage the kidney may include renal lipotoxicity, the direct and indirect effects of hyperinsulinaemia

as well as the adverse effects of adaptations to increased body mass, excretory load and sodium retention (Bagby, 2004).

In the present study, nephron endowment was not measured in adult offspring and is assumed to correspond to that observed in PN21 littermates. For this reason, total glomerular volume but not mean glomerular volume was quantified in 9 month old offspring. Total glomerular volume was similar in offspring of normal and high fat fed dams at 9 months of age, despite high fat diet exposed offspring having 20% more nephrons. This indicates that mean glomerular volume in the high fat offspring is less than in normal fat offspring. Together with the normal renal function and normal albumin excretion in these mice, this suggests they have a lower single nephron GFR.

Despite mild fasting hyperglycaemia, adult offspring of pre-gestational Type 2 diabetes displayed normal body weight, adiposity and glucose tolerance. Considering that offspring displayed enhanced nephron endowment and normal metabolic function in adulthood, it is not surprising that no long-term effects of maternal overnutrition on renal function or morphology were reported. Additional studies should focus on the metabolic and renal phenotype in aged offspring of high fat fed dams beyond 9 months of age. Experiments incorporating exposure of offspring to a second insult, such as a high fat feeding challenge or high salt diet challenge, are also required. Such chronic stressors may reveal a protective effect of enhanced nephron endowment or, conversely, may reveal a reduced functional renal reserve in offspring exposed to maternal high fat feeding.

3.6.6 Concluding remarks

In conclusion, offspring of dams fed a high fat diet had an elevated nephron endowment that was established relatively early in nephrogenesis. The increase in nephron number at E18.5 could not be attributed to maternal obesity or glucose intolerance in late pregnancy, which was similar in high fat and normal fat fed dams, but is most likely due to components of the maternal high fat diet promoting fetal and kidney growth in late gestation. Interestingly, ureteric branching morphogenesis and nephrogenesis were not altered at E15.5. This may be due to the differential transfer of nutrients across the placenta between mid to late gestation or may represent temporal changes in growth factor expression profiles within the embryonic kidney between E15.5 and E18.5. Further studies to assess the mechanisms of augmented nephrogenesis in maternal high fat feeding are required. As adults, offspring of high fat fed dams had normal renal function and, surprisingly, were not overweight or glucose intolerant. Additional studies incorporating a high fat feeding challenge in adult offspring are warranted. Despite an elevated nephron endowment, offspring of high fat fed dams may exhibit malformations in glomerular and nephron structure and a reduced functional renal reserve that is only unmasked in response to a chronic stressor.

CHAPTER FOUR

LEPTIN RECEPTOR DEFICIENT MICE AS A MODEL OF GESTATIONAL DIABETES – EFFECTS ON OFFSPRING KIDNEY DEVELOPMENT

4.1 Introduction

Gestational diabetes is defined as 'any degree of glucose intolerance with onset or first recognition during pregnancy' and by its definition includes women with diabetes preceding conception as well as women with pregnancy-associated glucose intolerance. Strikingly, gestational diabetes is reported in 5-17% of pregnancies (Templeton and Pieris-Caldwell, 2008, IADPSG Consensus Panel, 2010, Moses et al., 2011, DeSisto et al., 2014) with women of South Asian, East Asian and South-East Asian ethnicity at greatest risk for developing the condition. Other risk factors include advancing maternal age, overweight or obesity, family history of Type 2 diabetes, and previous pregnancies complicated by macrosomia or gestational diabetes (ADIPS, 2014). Recent changes in consensus guidelines are expected to further increase the number of women diagnosed with gestational diabetes (Moses et al., 2011, Sacks et al., 2012).

Gestational diabetes is typically asymptomatic for the mother but is associated with an increased life-time risk of Type 2 diabetes (Kim et al., 2002). Gestational diabetes poses a number of health implications for the offspring. Adverse perinatal outcomes such as birth weight above the 90th centile, Caesarean delivery, neonatal hypoglycaemia, birth injury and intensive neonatal care are increased in infants of gestational diabetic women (Shand et al., 2008, Catalano et al., 2012). Gestational diabetes is also associated with long-term consequences on offspring metabolic health, with offspring at an increased risk of obesity and glucose intolerance in later life (Boney et al., 2005, Clausen et al., 2008, Järvelin et al., 2009, Chandler-Laney et al., 2012).

There is also some evidence that gestational diabetes may perturb offspring kidney development. A high incidence of congenital abnormalities, particularly of the renal/urinary system, is reported in babies born to mothers with gestational diabetes. Infants have an increased risk of renal anomalies, urinary tract malformations and obstructive urinary defects, although it is important to acknowledge these studies may include women with unrecognised pre-existing Type 2 diabetes (Martínez-Frías et al., 1998, Aberg et al., 2001, Kamdem et al., 2005, Bánhidy et al., 2010, Shnorhavorian et al., 2011, Hsu et al., 2014). Recently, Cappuccini et al. (2013) reported reduced renal cortical volume, as a crude estimate of renal development and nephron endowment, in a small study of 3 year old children born to mothers with gestational diabetes. However, no human study has directly assessed nephron endowment in offspring born to mothers with gestational diabetes.

Experimental studies on the effects of gestational diabetes on kidney development are also limited. The majority of rodent studies investigating offspring kidney development and nephron endowment are reported in models of STZ-induced maternal diabetes. These publications cite a deficit in offspring nephron number but in the setting of severe fasting hyperglycaemia that in several studies is induced

prior to conception (Amri et al., 1999, Nehiri et al., 2008, Yan et al., 2014). STZ studies are also confounded by offspring growth restriction, which is not a common feature of human diabetic pregnancy (Tran et al., 2008, Hokke et al., 2013). Consequently, these findings are translatable only to women with uncontrolled Type 1 diabetes complicated by pre-eclampsia or vascular disease and not to women with pre-gestational diabetes or gestation diabetes. In Chapters 2 and 3, kidney development in a mouse model of diet-induced pre-gestational Type 2 diabetes was described. Offspring of dams with obesity and glucose intolerance before conception, which was surprisingly similar to controls in late gestation, were found to have increased nephron endowment. Importantly, the augmented nephron number was associated with maternal high fat feeding and not maternal glucose intolerance or overweight in pregnancy. Offspring kidney development in rodent models of gestational diabetes has not been previously investigated.

To improve our understanding of the effect of gestational diabetes on offspring kidney development, an animal model that better mimics the human condition is fundamental. The maternal phenotype should be characterised by mild-moderate hyperglycaemia, insulin resistance and postprandial hyperglycaemic excursions in late gestation, without the confounding effects of offspring growth restriction.

The *Lepr*$^{db/db}$ mouse is a genetic mouse model commonly used to study diabetes and obesity (Hummel et al., 1966, Coleman, 1978). Mice homozygous null for the *diabetes* allele (*db*) are defective in leptin receptor (*Lepr*) signalling causing an inability to suppress feeding behaviour (Chen et al., 1996). *Lepr*$^{db/db}$ mice exhibit hyperinsulinaemia and obesity by 2-4 weeks of age and profound hyperglycaemia by 6 weeks. While homozygous null mutant females are sterile, female mice heterozygous for the mutant allele (*Lepr*$^{db/+}$) are fertile. The literature describes female *Lepr*$^{db/+}$ mice with normal glucose and insulin levels in the non-pregnant state, yet during pregnancy these mice display a phenotype of gestational diabetes (Kaufmann et al., 1981, Ishizuka et al., 1999, Yamashita et al., 2003, Stanley et al., 2011). Pregnant *Lepr*$^{db/+}$ dams exhibit hyperphagia, increased weight gain, moderate glucose intolerance and hyperinsulinaemia in late gestation compared with wild type mice. Moreover, there is an increased prevalence of neonatal macrosomia, with offspring weighing 5-10% more than controls (Kaufmann et al., 1981, Lawrence et al., 1989, Yamashita et al., 2001, Yamashita et al., 2003, Lambin et al., 2007, Stanley et al., 2011).

Gestational diabetes is a condition associated with underlying defects in insulin secretion and sensitivity that are unmasked in response to the metabolic stressors of pregnancy (Kim, 2014). In pregnant *Lepr*$^{db/+}$ mice, glucose intolerance ultimately arises from a genetic susceptibility uncovered by pregnancy-induced metabolic alterations. While human mutations in leptin and the leptin receptor

are rare, the metabolic phenotype reported in pregnant *Lepr*$^{db/+}$ mice is comparable to that reported in women with gestational diabetes. The *Lepr*$^{db/+}$ mouse therefore provides a suitable model to study the effect of gestational diabetes on offspring kidney development.

This chapter is presented in three sections. Section A presents preliminary data evaluating pregnant *Lepr*$^{db/+}$ mice for hyperphagia, weight gain and glucose intolerance. Section B describes the genetic deletion of *misty*, a confounding genetic mutation maintained in linkage disequilibrium with *diabetes*, from the *Lepr*$^{db/+}$ colony and presents preliminary data of glucose intolerance and weight gain in pregnant *Lepr*$^{db/+}$ dams without *misty*. Section C presents complete analysis of the maternal phenotype in *Lepr*$^{db/+}$ dams following the removal of *misty*, and kidney development in offspring of *Lepr*$^{db/+}$ dams.

4.2 CHAPTER 4A: The *Lepr*$^{db/+}$ *misty* mouse as a model of gestational diabetes; A preliminary study

4.2.1 CHAPTER 4A: Introduction

Several studies have identified the leptin receptor deficient mouse, *Lepr*$^{db/+}$, as a model of gestational diabetes (Kaufmann et al., 1981, Ishizuka et al., 1999, Yamashita et al., 2001, Shao et al., 2002, Yamashita et al., 2003, Stanley et al., 2011). In these studies, *Lepr*$^{db/+}$ mice are reported to spontaneously develop glucose intolerance during gestation and exhibit hyperphagia, increased weight gain and macrosomic offspring compared with wild type mice. Chapter 4A describes the establishment of a colony of *Lepr*$^{db/+}$ mice at Monash University and the pilot studies performed to characterise the gestational diabetes phenotype as previously described in the literature.

4.2.2 CHAPTER 4A: Aim

- To evaluate the leptin receptor deficient mouse (*Lepr*$^{db/+}$) as a model of gestational diabetes.

4.2.3 CHAPTER 4A: Hypothesis

- Leptin receptor deficient mice will exhibit hyperphagia, increased weight gain and impaired glucose tolerance in late pregnancy and will thereby provide a clinically relevant model of gestational diabetes.

4.2.4 CHAPTER 4A: Methods

4.2.4.1 Animal husbandry

All animal handling and experimental protocols were approved by the Animal Ethics Committee of Monash University (MARP/2011/122) and conformed to the guidelines of the National Health and Medical Research Council of Australia. Two breeding pairs of C57BLKS.Cg-*Dock7*^{m} +/+ *Lepr*^{db} /J mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA) at 6 weeks of age, and a colony of mice was established at Animal Research Laboratories, Monash University (MARP/2011/114/BC). Mice were housed in Optiplex cages with sawdust bedding in a controlled environment (20-22°C, 11-45% humidity) with a 12/12-hour light-dark cycle. Mice had *ad libitum* access to water and standard chow (Barastoc; Ridley AgriProducts, Pakenham, Victoria, Australia).

4.2.4.2 The C57BLKS.Cg-Dock7m +/+ *Lepr*^{db} /J mouse

C57BLKS.Cg-*Dock7*^{m} +/+ *Lepr*^{db} /J mice are repulsion double heterozygote nulls for *Dock7*^{m} and *Lepr*^{db}. These alleles are maintained in linkage disequilibrium on chromosome 4. The *diabetes* (*db*) mutation arose spontaneously in a colony of C57BLKS/J inbred mice at The Jackson Laboratory (Hummel et al., 1966), and was later identified as a single point mutation in the leptin receptor (*Lepr*) (Chen et al., 1996). A G-to-T transversion in this allele creates a donor splice site and abnormal splicing, causing a

106 nucleotide insertion in the long-form transcript and the premature termination of the intracellular domain necessary for signal transduction (Chen et al., 1996). The *Dock7* (dedicator of cytokinesis protein 7) gene is tightly linked to *Lepr*, and the *misty* (*m*) mutation was originally incorporated into stock at The Jackson Laboratory to identify mice wild type for the $Lepr^{db}$ allele. $Dock7^{m}$ is a recessive mutation that leads to a dilution of coat colour (mice have light grey fur) and a white tail tip. In the present study, C57BLKS.Cg-$Dock7^{m}$ +/+ $Lepr^{db}$ /J mice (repulsion double heterozygotes; hereafter referred to as $Lepr^{db/+}$ *misty*) were used to model gestational diabetes. C57BLKS.Cg-$Dock7^{m}$ +/$Dock7^{m}$ + /J mice (wild type for *db*, recessive for *misty*; hereafter referred to as +/+ *misty*) were used as control dams.

4.2.4.3 Genotyping for the $Lepr^{db}$ mutation

A modified version of Horvat and Bunger's PCR reaction fragment length polymorphism assay was used to identify the $Lepr^{db}$ mutation (Horvat and Bünger, 1999). This protocol uses modified primers to create two restriction sites that can be cut by the AccI restriction enzyme – one distinguishing between the mutant and wild type *Lepr* allele, and the other serving as a digestion control site for both wild type and mutant alleles.

DNA was extracted and amplified from tail tissue using RED Extract-N-Amp Tissue PCR kit (Sigma Aldrich, Castle Hill, NSW, Australia). For DNA extraction, 100 µl of extraction solution and 25 µl of tissue preparation solution were added to each tail sample and incubated at room temperature for 10 minutes, and then at 95°C for 3 minutes. 100 µl of neutralisation solution was added to each digested sample and the solution was vortexed. For the PCR reaction, 1 µl of extract was added to 3 µl of water, 10 µl RED extract-N-Amp PCR reaction mix, 0.5 µl *db* Acc forward primer and 0.5 µl *db* Acc reverse primer to yield a total reaction volume of 15 µl (

Table 4.1 and **Table 4.2**). Amplification was carried out in a Biorad iCycler Thermal Cycler using a PCR profile of: 1 cycle at 94°C for 2 minutes for initial denaturation; 35 cycles of 30 seconds at 94°C to denature, 30 seconds at 55°C to anneal and 30 seconds at 72°C to extend; followed by a final extension at 72°C for 7 minutes (**Table 4.3**). Products were incubated with 0.6 µl AccI restriction enzyme (New England Biolabs, Ipswich, MA, USA) and 1.5 µl enzyme buffer at 37°C for 8 hours. Digests were then analysed in a 3.5% agarose gel (analytical grade agarose (Promega, San Luis Obispo, CA, USA) and 1xTris-Borate-EDTA (TBE) buffer (Sigma Aldrich, Castle Hill, NSW, Australia) containing Gel Red (1 µl per 100 ml; Biotium, Hayward, CA, USA)). Samples with 85- and 24-bp fragments were identified as *Lepr*$^{+/+}$ and samples with 85-, 58-, 27- and 24-bp fragments were identified as *Lepr*$^{db/+}$ (**Figure 4.1**).

Table 4.1 PCR amplification - reaction volume specifications

	Volume per sample (µl)
Water, milliQ autoclaved	3
RED extract-N-Amp PCR reaction mix	10
db Acc Forward Primer	0.5
db Acc Reverse Primer	0.5
DNA (solution from tissue extraction)	1
Total volume	15

Table 4.2 PCR amplification - primers

db Acc Forward Primer	5' ATG ACC ACT ACA GAT GAA CCC AGT CTA C 3' (28 bases)
db Acc Reverse Primer	5' CAT TCA AAC CAT AGT TTA GGT TTG TCT 3' (27 bases)

Table 4.3 PCR amplification - thermal cycler conditions

Step	Temperature	Time	Cycles
Initial denaturation	94°C	2 minutes	1
Denaturation	94°C	30 seconds	35
Annealing	55°C	30 seconds	
Extension	72°C	30 seconds	
Final extension	72°C	7 minutes	1
Hold	15°C	∞	

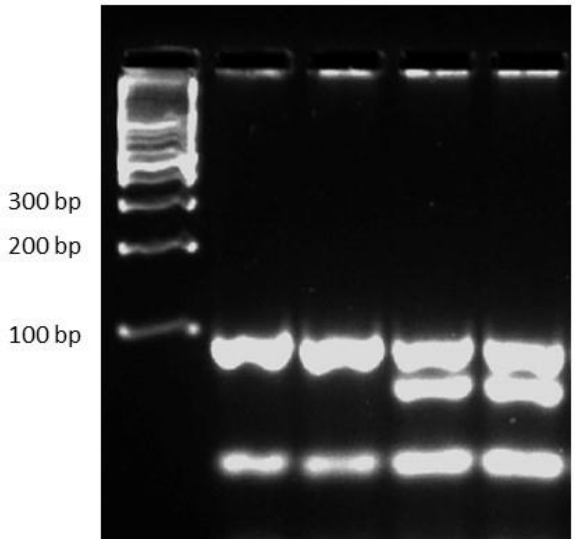


Figure 4.1 Gel electrophoresis of tissue samples distinguishing *Lepr*$^{db/+}$ and *Lepr*$^{+/+}$ alleles
Lane 1: 100bp DNA ladder. Lane 2 and 3: *Lepr*$^{+/+}$ identified by 85- and 24-bp bands. Lane 4 and 5: *Lepr*$^{db/+}$ identified by 85-, 58-, 27- and 24-bp fragments.

4.2.4.4 Mating protocol

Female *Lepr*$^{db/+}$ *misty* and +/+ *misty* mice between 8-14 weeks of age were mated overnight with male +/+ *misty* mice. The following morning females were checked for the presence of a vaginal seminal plug. The presence of a vaginal plug confirmed that mating had taken place and indicated E0.5 of pregnancy.

4.2.4.5 Maternal body weight and food intake

Animal weight was recorded at time of positive vaginal plug and throughout pregnancy. Gestational weight gain was calculated by subtracting the weight of dams at E0.5 from their weight at E17.5. Food intake was measured throughout pregnancy. Dams of the same genotype were caged individually or in pairs. Every 2-3 days the weight of standard chow removed from the food hopper and the weight of food left on the base of the cage was recorded and used to calculate food consumption per mouse. Total food intake was calculated for the period E0.5 to E17.5.

4.2.4.6 Glucose tolerance test

Mice underwent a GTT at E17.5 of pregnancy. GTTs were performed as previously described (**Section 2.4.4**).

4.2.4.7 Statistical analysis

Data were analysed using SPSS software and GraphPad Prism software. Maternal weights and food intake were analysed by independent samples t-test. GTTs were analysed by repeated measures ANOVA. A glucose AUC value was calculated using GraphPad and analysed by independent samples t-test. Data were tested for normality using a D'Agostino-Pearson omnibus normality test. Data are presented as mean ± SEM. $P<0.05$ was considered statistically significant.

4.2.5 CHAPTER 4A: Results

4.2.5.1 *Lepr*$^{db/+}$ *misty* mice are heavier than +/+ *misty* mice but exhibit normal weight gain and food intake during pregnancy

Maternal weight was measured at E0.5 and E17.5. Compared to +/+ *misty* dams, *Lepr*$^{db/+}$ *misty* mice were significantly heavier at E0.5 (24% heavier; $P<0.0001$) and at E17.5 (14% heavier; $P=0.0007$). Weight gain and food intake, measured between E0.5 and E17.5, were similar in the two groups (**Table 4.4)**.

Table 4.4 Maternal weight, weight gain and food intake in +/+ *misty* and *Lepr$^{db/+}$ misty* dams

	+/+ misty (*n*)	***Lepr$^{db/+}$ misty*** (*n*)	***P***
Weight E0.5, g	19.0 ± 0.2 (10)	23.5 ± 0.6 (9)	**<0.0001**
Weight E17.5, g	33.6 ± 0.8 (10)	38.2 ± 0.7 (9)	**0.0007**
Pregnancy weight gain, g	14.6 ± 0.8 (10)	14.7 ± 0.7 (9)	0.954
Pregnancy food intake, g	58.9 ± 1.8 (6)	59.6 ± 2.2 (8)	0.815

Analysis by independent samples t-test. Values are mean ± SEM.

4.2.5.2 *Lepr$^{db/+}$ misty* dams have variable penetrance of glucose intolerance

Mice underwent a GTT at E17.5. As shown in **Figure 4.2A**, there was a slight elevation in blood glucose levels 30 minutes after a glucose load in *Lepr$^{db/+}$ misty* dams, but no marked change in subsequent measures of glucose or fasting glucose concentrations. A repeated measures ANOVA revealed no statistical difference in glucose tolerance between *Lepr$^{db/+}$ misty* and +/+ *misty* dams (*P*=0.175). Upon further investigation of individual glucose tolerance curves of *Lepr$^{db/+}$ misty* dams, it was identified that three out of nine dams had substantially elevated blood glucose levels 30 minutes after a glucose load (**Figure 4.2B**) indicating a variable penetrance of impaired glucose tolerance in *Lepr$^{db/+}$ misty* dams. Glucose AUC was similar between all *Lepr$^{db/+}$ misty* and +/+ *misty* dams (+/+ *misty* 907.0 ± 17.5 mmol/l.min, *Lepr$^{db/+}$ misty* 966.9 ± 57.0 mmol/l.min; *P*=0.307; **Figure 4.3**).

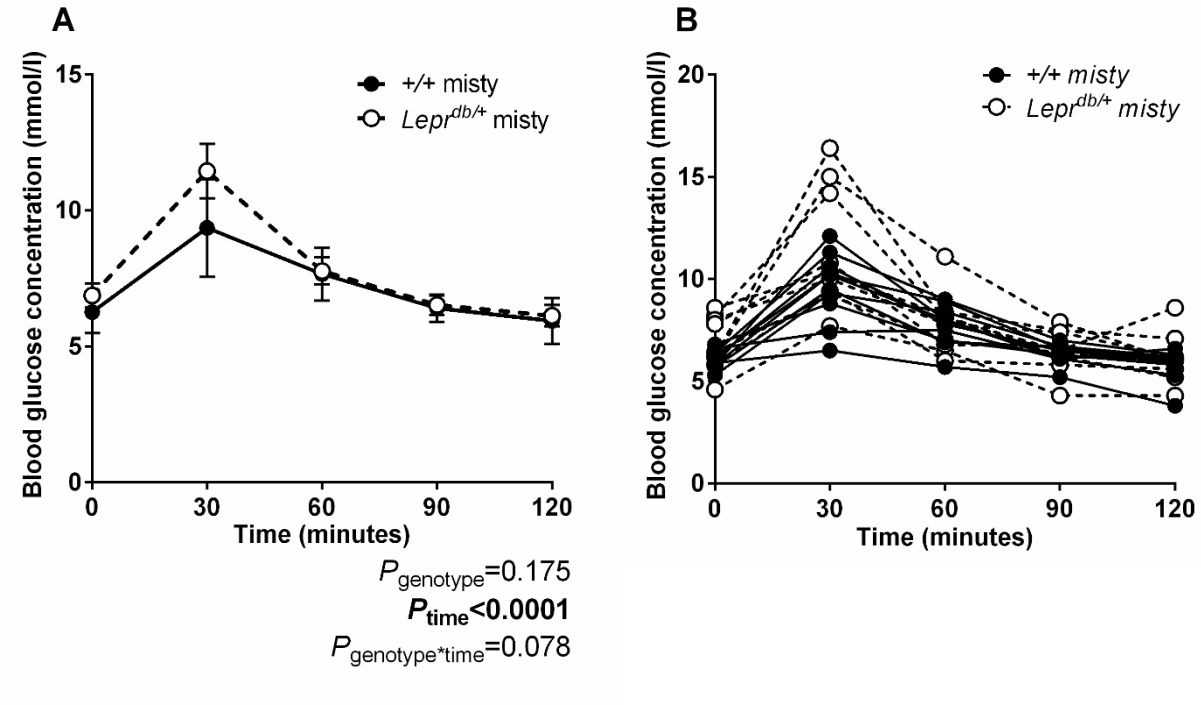


Figure 4.2 Glucose tolerance tests in +/+ *misty* and *Lepr$^{db/+}$ misty* dams at E17.5

A Mean GTT curves and **B** individual GTT curves in +/+ *misty* and *Lepr$^{db/+}$ misty* mice at E17.5. GTT analysed by repeated measures ANOVA for maternal genotype and time. Values are mean ± SEM. *n*(+/+ *misty*)=10, *n*(*Lepr$^{db/+}$ misty*)=9.

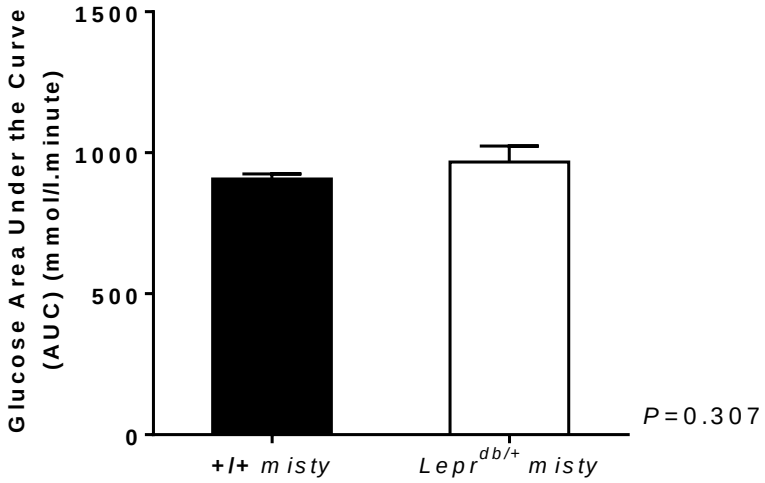


Figure 4.3 Glucose AUC of +/+ *misty* and *Lepr$^{db/+}$ misty* dams at E17.5
Analysis by independent samples t-test. Values are mean ± SEM. *n*(+/+ *misty*)=10, *n*(*Lepr$^{db/+}$ misty*)=9.

4.2.5.3 Offspring of *Lepr$^{db/+}$ misty* dams have normal body weight at PN2

Dams were left to litter down and litters were assessed at PN2. Litter size was significantly greater in *Lepr$^{db/+}$ misty* dams compared with +/+ *misty* dams (+/+ *misty* 5.6 ± 0.5 pups, *n*=10 litters; *Lepr$^{db/+}$ misty* 7.2 ± 0.4 pups, *n*=9 litters; *P*=0.013). There was no difference in offspring body weight at PN2 (+/+ *misty* 1.69 ± 0.07 g, *Lepr$^{db/+}$ misty* 1.55 ± .07 g; *P*=0.166; **Figure 4.4**).

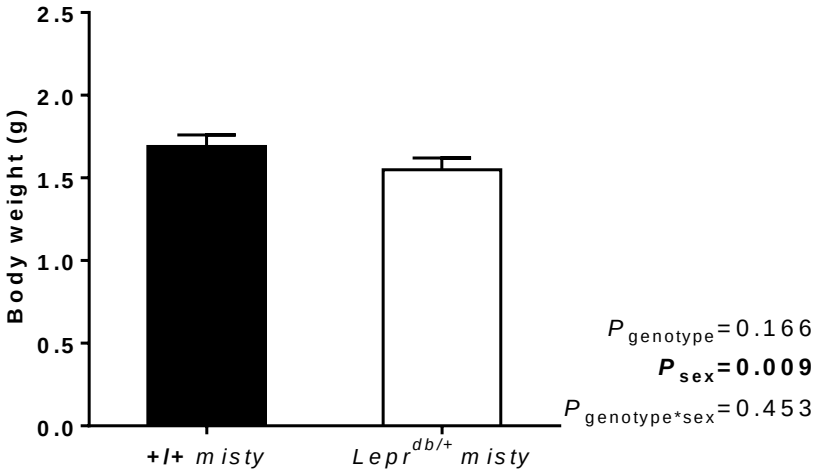


Figure 4.4 Offspring body weight of +/+ *misty* and *Lepr$^{db/+}$ misty* dams at PN2
Body weight of pups of +/+ *misty* and IGT *Lepr$^{db/+}$ misty* dams. Analysis by two-way ANOVA with maternal genotype and sex as variables, weighted for litter using a linear mixed model. Values are mean ± SEM. *n*(+/+ *misty*)=58 pups from 10 litters, *n*(IGT *Lepr$^{db/+}$ misty*)=66 pups from 9 litters.

4.2.6 CHAPTER 4A: Conclusion

Analysis of *Lepr*$^{db/+}$ *misty* dams revealed no difference in food intake or weight gain compared to +/+ *misty* dams during pregnancy. GTTs at E17.5 showed no overall difference in glucose profiles between groups. However, upon evaluation of individual GTTs, a proportion of *Lepr*$^{db/+}$ *misty* dams (three out of nine) were found to have substantially elevated blood glucose levels 30 minutes after a glucose load, indicating a variable penetrance of impaired glucose tolerance in *Lepr*$^{db/+}$ *misty* dams. Furthermore, offspring macrosomia was not observed. These results indicate that *Lepr*$^{db/+}$ *misty* mice do not consistently model gestational diabetes when compared to the +/+ *misty* mouse.

At this stage of experimentation, concerns were raised regarding the potentially confounding effect of *misty* on growth traits. Previously, Truett et al. (1998) found C57BL6/J *misty* mice (homozygous for the *Dock7*^{m} mutation) to be significantly shorter, weigh less and have reduced adipose tissue mass than C57BL6/J mice (wild type for *misty*) in the absence of *Lepr*^{db}. The authors concluded that the *misty* mutation should be removed from *Lepr*^{db} populations.

The first report describing the use of *Lepr*$^{db/+}$ dams as a model of gestational diabetes used wild type *misty* littermates as controls (Kaufmann et al., 1981). Three of the subsequent papers that use this mouse model specifically mention the incorporated *misty* gene (Lawrence et al., 1989, Lambin et al., 2007, Bobadilla et al., 2010). Unfortunately, two of these publications did not report maternal glucose levels (Lawrence et al., 1989, Lambin et al., 2007) whilst the third publication described no difference in glucose tolerance between groups (Bobadilla et al., 2010). Harrod et al. (2011) recognised the limitations of the *misty* mouse and used C57BL6/J mice as controls rather than *misty* littermates of *Lepr*$^{db/+}$ mice, and also found no difference in glucose tolerance. A further five studies that utilised the *Lepr*$^{db/+}$ mouse as a model of gestational diabetes make no mention of *misty* but cite the use of *Lepr*$^{db/+}$ animals purchased from The Jackson Laboratory on the C57BLKS/J background, the line on which *misty* was originally incorporated (Ishizuka et al., 1999, Yamashita et al., 2001, Shao et al., 2002, Yamashita et al., 2003, Stanley et al., 2011). These studies show elevated food intake and weight gain as well as raised fasting glucose and impaired glucose tolerance in pregnant *Lepr*$^{db/+}$ dams compared with control dams.

Dock7 encodes a widely expressed Rho family guanine nucleotide exchange factor that contributes to a range of cellular signalling events (Blasius et al., 2009). The *misty* mutation leads to the premature termination of this protein, and is known to affect adiposity and growth and may have additional undesirable physiological effects. To circumvent the potentially confounding effect of *misty* and the inconsistent phenotype of gestational diabetes using *Lepr*$^{db/+}$ *misty* dams described in Chapter 4A,

standard C57BLKS/J mice (without *misty*) were purchased from The Jackson Laboratory for the purpose of strategic breeding to remove the *misty* mutation from the *Lepr*$^{db/+}$ colony (Chapter 4B).

4.3 CHAPTER 4B: The *Lepr*$^{db/+}$ mouse as a model of gestational diabetes; A preliminary study

4.3.1 CHAPTER 4B: Introduction

This chapter describes the breeding strategy used to remove *misty* from the *Lepr*$^{db/+}$ colony and presents preliminary data on the subsequent characterisation of this mouse line to determine whether it presents as an ideal model of gestational diabetes.

4.3.2 CHAPTER 4B: Aim

- To evaluate, following the removal of *misty*, the leptin receptor deficient mouse as a model of gestational diabetes.

4.3.3 CHAPTER 4B: Hypothesis

- Leptin receptor deficient mice (wild type for *misty*) will exhibit hyperphagia, increased weight gain and impaired glucose tolerance in late pregnancy and will thereby provide a clinically relevant model of gestational diabetes.

4.3.4 CHAPTER 4B: Methods

4.3.4.1 Breeding strategy to remove *misty*

In the original C57BLKS.Cg-*Dock7*^{m} +/+ *Lepr*^{db} /J mouse, the diabetes and *misty* mutations are tightly linked and maintained in repulsion, with each homologous chromosome having one mutant and one wild type allele. Due to the allelic arrangement of the two linked heterozygous loci, an additional mouse line was required to breed out *misty*. Standard C57BLKS/J mice were purchased from The Jackson Laboratory and bred with *Lepr*$^{db/+}$ *misty* mice. This generated mice that genotyped wild type for *Lepr*^{db} and assumed heterozygous for *misty,* and were subsequently disregarded. This breeding strategy also generated mice that genotyped heterozygous for *Lepr*^{db} and assumed wild type for *misty*. These mice were used for experimentation. It should be noted that *misty* was not genotyped for and mice were assumed to be non-recombinant, however there was a 1% possibility of recombination (JAX, 2015).

A schematic of the original and new breeding strategies is presented in **Figure 4.5**. All subsequent experiments presented in Chapter 4B and 4C were conducted using *Lepr*$^{db/+}$ *Dock7*$^{+/+}$ mice to model gestational diabetes (hereafter abbreviated as *Lepr*$^{db/+}$), with C57BLKS/J mice used as control mice (hereafter abbreviated as +/+).

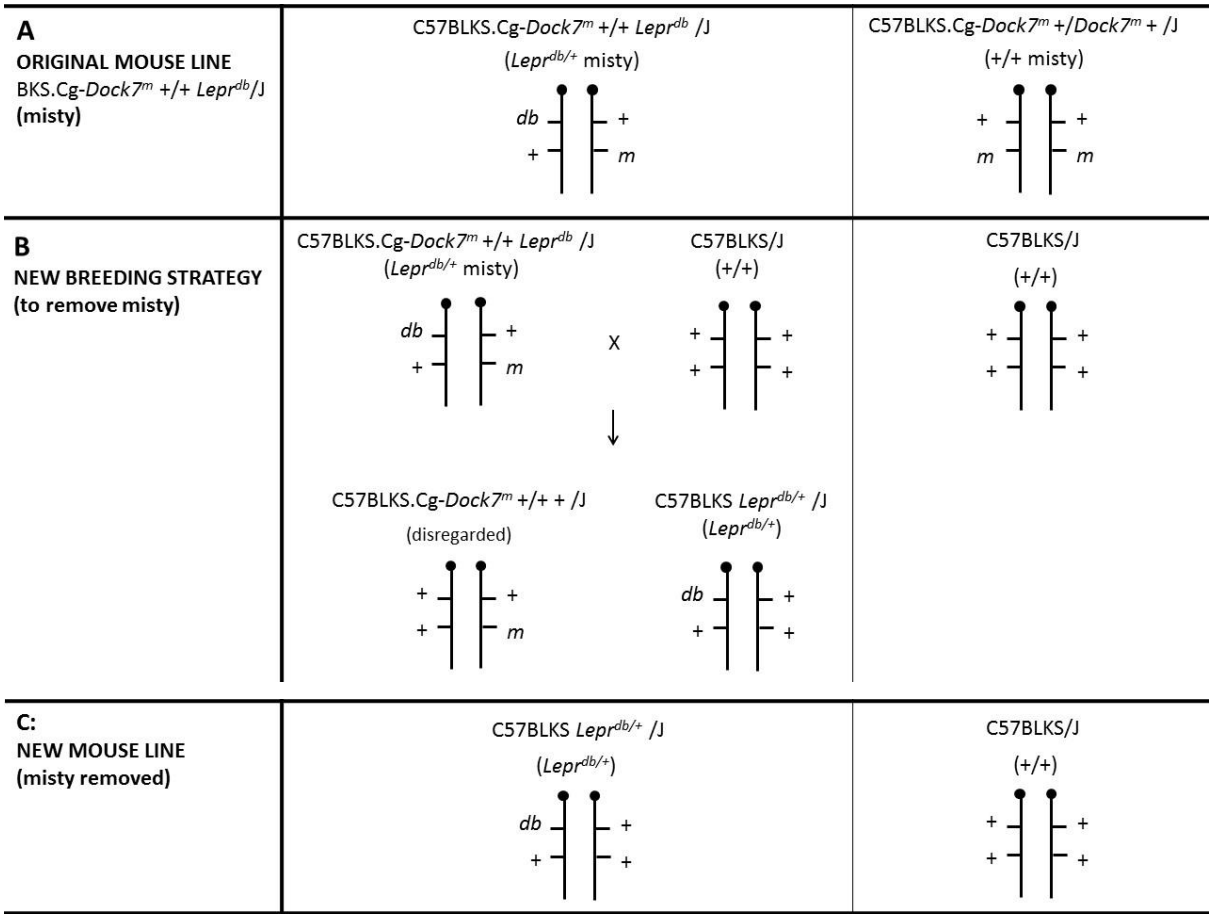


Figure 4.5 Schematic of the original and new breeding strategies

A Breeding strategy of the original *misty* mouse line. Originally, heterozygote mice carrying both diabetes and *misty* mutations in repulsion were used to model gestational diabetes (top left panel). Originally, control mice were wild type for *db* and homozygous mutant for *misty* (top right panel). **B** To remove *misty* from the colony, standard C57BLKS/J mice were bred with $Lepr^{db/+}$ *misty* mice (middle left panel). Heterozygote *db* mice generated from these matings were considered wild type for *misty* and used to model gestational diabetes in all subsequent experiments. Standard C57BLKS/J mice were used as control mice instead of +/+ *misty* mice (middle right panel). **C** Summary of the new $Lepr^{db/+}$ mouse line following the removal of *misty* from the colony (bottom left panel) and the C57BLKS/J (+/+) mouse line (bottom right panel).

4.3.4.2 Mating protocol

Female $Lepr^{db/+}$ and +/+ mice between 8 and 14 weeks of age were mated overnight with male +/+ mice. The following morning females were checked for the presence of a vaginal seminal plug indicating E0.5 of pregnancy.

4.3.4.3 Maternal body weight

Animal weight was recorded at time of positive vaginal plug and throughout pregnancy. Gestational weight gain was calculated as described in **Section 4.2.4.5**.

4.3.4.4 Glucose tolerance test

Mice underwent a GTT at E17.5 as previously described in **Section 2.4.4**.

4.3.4.5 Statistical Analysis

Data were analysed as previously described (**Section 4.2.4.7**).

4.3.5 CHAPTER 4B: Results

4.3.5.1 *Lepr*$^{db/+}$ dams are heavier and have greater pregnancy weight gain than +/+ dams

As shown in **Table 4.5**, the cohort of *Lepr*$^{db/+}$ dams were significantly heavier at E0.5 (22% heavier; *P*=0.001) and E17.5 (21% heavier; *P*=0.0003) than +/+ dams. Gestational weight gain was greater in *Lepr*$^{db/+}$ dams (*P*=0.005), yet was also associated with an increase in litter size (*P*=0.036).

Table 4.5 Maternal body weight and weight gain in the first cohort of +/+ and *Lepr*$^{db/+}$ dams

	+/+ (*n*)	***Lepr*$^{db/+}$** (*n*)	***P***
Body weight E0.5, g	19.9 ± 0.6 (8)	24.3 ± 0.8 (7)	**0.001**
Body weight E17.5, g	32.4 ± 1.2 (8)	39.2 ± 0.7 (7)	**0.0003**
Pregnancy weight gain, g	12.4 ± 0.6 (8)	14.9 ± 0.5 (7)	**0.005**
Litter size	6.1 ± 0.5 (8)	7.7 ± 0.3 (7)	**0.036**

Analysis by independent samples t-test. Values are mean ± SEM.

4.3.5.2 Penetrance of maternal glucose intolerance is not improved in *Lepr*$^{db/+}$ dams

Repeated measures ANOVA revealed no statistical difference in glucose tolerance between *Lepr*$^{db/+}$ and +/+ dams at E17.5 (*P*=0.234; **Figure 4.6**). There was also no difference in glucose AUC between +/+ and *Lepr*$^{db/+}$ dams (+/+ 799.7 ± 17.5 mmol/l.min, *Lepr*$^{db/+}$ 881.8 ± 65.0 mmol/l.min; *P*=0.217). When plotted as individual glucose tolerance curves, it was found that one out of seven *Lepr*$^{db/+}$ dams had a markedly elevated peak glucose level (**Figure 4.7A**). When compared to the mean glucose profile of +/+ dams, it was revealed that an additional three dams had elevated peak glucose levels and three dams had lower peak glucose levels (**Figure 4.7B**).

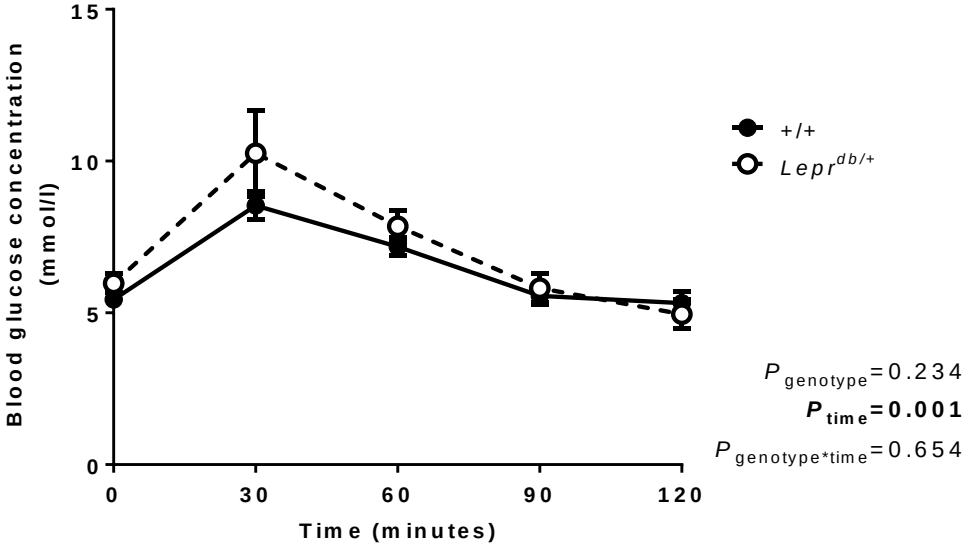


Figure 4.6 Glucose tolerance tests in +/+ and *Lepr*$^{db/+}$ mice at E17.5
Mean GTT curves of +/+ and *Lepr*$^{db/+}$ mice at E17.5. GTT analysed by repeated measures ANOVA for maternal genotype and time. Values are mean ± SEM. *n*(+/+)=8, *n*(*Lepr*$^{db/+}$)=7.

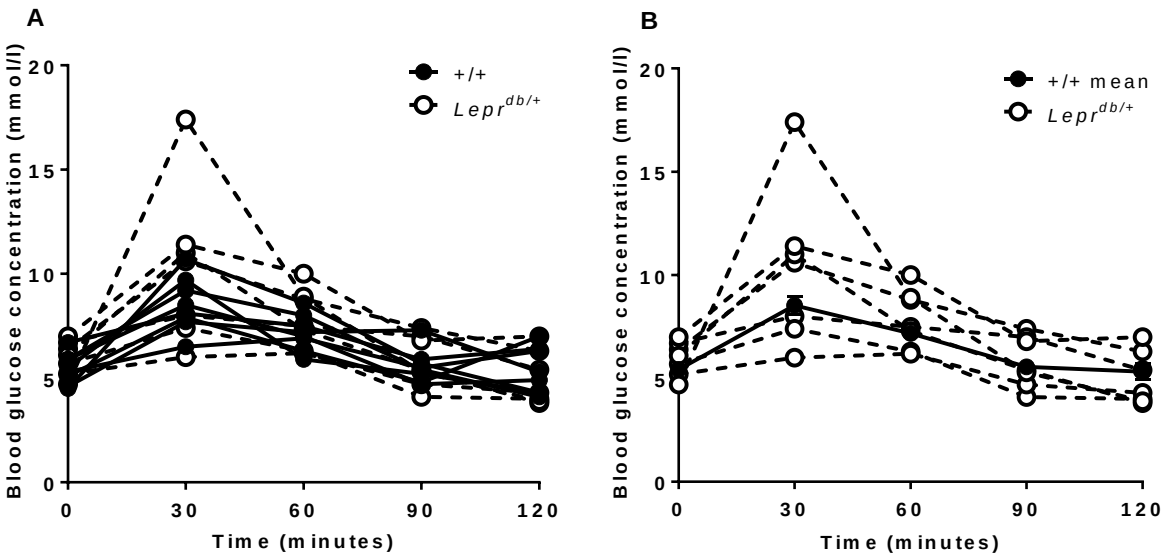


Figure 4.7 Individual glucose tolerance tests in +/+ and *Lepr*$^{db/+}$ mice at E17.5
A Individual GTT curves of +/+ and *Lepr*$^{db/+}$ mice at E17.5. **B** Mean GTT curve of +/+ mice and individual GTT curves of *Lepr*$^{db/+}$ dams at E17.5. *n*(+/+)=8, *n*(*Lepr*$^{db/+}$)=7.

4.3.6 CHAPTER 4B: Conclusion

The removal of *misty* was required to eliminate the potentially confounding effects of this allele on growth.

Following the elimination of *misty, Lepr*$^{db/+}$ dams exhibited a greater gestational weight gain than +/+ dams, a phenotype that was not present prior to the removal of *misty*. Elevations in gestational weight gain, and associated increases in food intake, have been reported previously in *Lepr*$^{db/+}$ dams modelling gestational diabetes (Ishizuka et al., 1999, Yamashita et al., 2001, Yamashita et al., 2003, Lambin et al., 2007). Unfortunately food intake was not measured in the present study and therefore cannot be correlated with the observed increase in gestational weight gain. It is important to note that litter size was also increased in *Lepr*$^{db/+}$ dams and may contribute to and therefore confound, the increase in gestational weight gain observed.

Unfortunately the removal of *misty* did not improve the prevalence of glucose intolerance in *Lepr*$^{db/+}$ dams. Of the seven tested *Lepr*$^{db/+}$ dams in this preliminary study, only one exhibited marked glucose intolerance and an additional three showed only a slight elevation in glucose levels in a GTT. All subsequent experiments were therefore conducted using +/+ and *Lepr*$^{db/+}$ mice, without *misty*, with the recognition that this mouse line does not produce a consistent phenotype of impaired glucose tolerance. Full analysis of the maternal phenotype of *Lepr*$^{db/+}$ dams and their offspring is presented in Chapter 4C.

4.4 CHAPTER 4C: A detailed analysis of *Lepr*$^{db/+}$ mice as a model of gestational diabetes and the effect on offspring kidney development

4.4.1 CHAPTER 4C: Introduction

Preliminary analyses of pregnant *Lepr*$^{db/+}$ *misty* and *Lepr*$^{db/+}$ mice were described in Chapter 4A and 4B. GTTs performed in late gestation revealed incomplete penetrance of glucose intolerance with elevated glucose profiles seen in approximately half of *Lepr*$^{db/+}$ dams compared with +/+ dams. These findings were unexpected and were not been reported in previous studies (Kaufmann et al., 1981, Ishizuka et al., 1999, Yamashita et al., 2001, Yamashita et al., 2003, Stanley et al., 2011).

Despite incomplete penetrance, analysis of *Lepr*$^{db/+}$ mice as a model of gestational diabetes was continued with the acknowledgement that this mouse line does not produce a consistent phenotype of glucose intolerance in pregnancy. A detailed analysis of glucose tolerance and body weight in selected *Lepr*$^{db/+}$ mice before pregnancy and during gestation and lactation is presented below. Offspring of selected *Lepr*$^{db/+}$ dams were assessed to investigate the effects of maternal glucose intolerance on growth parameters and kidney development.

4.4.2 CHAPTER 4C: Aims

- To characterise the maternal phenotype of leptin receptor deficient (*Lepr*$^{db/+}$) mice as a model of gestational diabetes.
- To assess growth and kidney development in embryonic and early postnatal offspring of *Lepr*$^{db/+}$ dams.

4.4.3 CHAPTER 4C: Hypotheses

- *Lepr*$^{db/+}$ dams will exhibit normal body weight and glucose tolerance prior to pregnancy.
- In late gestation *Lepr*$^{db/+}$ dams will exhibit incomplete penetrance of glucose intolerance, elevated weight gain and hyperphagia to model gestational diabetes.
- Offspring of glucose intolerant *Lepr*$^{db/+}$ dams will be macrosomic and have a deficit in nephron endowment relative to body weight.

4.4.4 CHAPTER 4C: Methods

4.4.4.1 Animal husbandry

All animal handling and experimental protocols were approved by the Animal Ethics Committee of Monash University (MARP/2011/122) and conformed to the guidelines of the National Health and Medical Research Council of Australia. Colonies of *Lepr*$^{db/+}$ and +/+ mice were maintained at Animal Research Laboratories, Monash University. Mice were housed in Optiplex cages with sawdust bedding

in a controlled environment (20-22°C, 11-45% humidity) with a 12/12-hour light-dark cycle. Mice had *ad libitum* access to water and standard chow.

4.4.4.2 Mating protocol

Female *Lepr*$^{db/+}$ and +/+ mice between approximately 8-14 weeks of age were mated overnight with male +/+ mice. The presence of a vaginal plug confirmed that mating had taken place and indicated E0.5 of pregnancy.

4.4.4.3 Weight and food intake

Weight gain and food intake were recorded from E0.5 to E17.5 of pregnancy, as described **in Section 4.2.4.5**.

4.4.4.4 Glucose tolerance test

GTTs were performed at E17.5 in all *Lepr*$^{db/+}$ and +/+ mice, as described in **Section 2.4.4**. Selected mice also underwent GTTs prior to pregnancy (typically performed the day prior to mating), at E12.5, and on PN4 and PN21.

4.4.4.5 Plasma analyses

Maternal and fetal plasma insulin and leptin protein content were quantified by ELISA. A cohort of *Lepr*$^{db/+}$ and +/+ dams were collected at E17.5 after a GTT following 6 hours fasting.

Pregnant dams were anaesthetised using Isoflurane in an induction chamber. When unresponsive (absence of pedal reflex) an incision was made along the thoracic wall and through the diaphragm and ribcage to expose the heart. Maternal blood was collected from the left ventricle using a 26-gauge needle with syringe. Approximately 500-1000 µl of blood was collected into an Eppendorf tube containing EDTA to prevent coagulation. Blood was centrifuged for 10 minutes at 4°C at 3000 rpm. Maternal plasma was aspirated from packed cells and frozen at -80°C.

The incision was next extended along the abdominal wall to expose the uterine horns, which were excised. Fetuses were decapitated and 10-50 µl of fetal blood was collected. Fetal blood was centrifuged for 10 minutes at 4°C at 3000 rpm, and fetal plasma was aspirated from packed cells and frozen at -80°C. Tails of offspring of *Lepr*$^{db/+}$ dams were collected and frozen for genotyping (as described in **Section 4.2.4.3**).

Maternal and fetal plasma insulin and leptin protein content were quantified as previously described in **Section 2.4.8 and Section 3.4.2**. Due to small volumes, fetal plasma samples were pooled. Data are presented per litter, where each pooled sample represents *n*=1. Only plasma of +/+ offspring of *Lepr*$^{db/+}$ dams were analysed.

4.4.4.6 Tissue collection for analysis of offspring kidney development

Offspring of *Lepr*$^{db/+}$ and +/+ dams were collected at two time points for analysis of kidney development: E18.5 and PN21. Embryos were collected at E18.5 for estimation of nephron number as this is the latest time point to assess nephrogenesis before birth and enabled assessment of the maximal effect of the maternal environment on kidney development. Offspring were collected at PN21 to estimate final nephron endowment (nephrogenesis is complete by approximately PN5 in the mouse). This time point was also chosen as the kidney has distinctive medullary and cortical zones.

To avoid the confounding effect of genotype on offspring development, only +/+ offspring of Lepr$^{db/+}$ dams were analysed.

4.4.4.6.1 E18.5 tissue collection

Tissue was collected at E18.5 as previously described (**Section 3.4.1.1**). Briefly, pregnant dams were anaesthetised and embryos removed by Caesarean section. Placentae and embryos were weighed and measurements of fetal crown-rump length and head diameter were recorded. Maternal and fetal blood was collected and plasma was stored at -80°C. With the aid of a dissecting microscope, embryos were sexed and the kidneys were carefully excised and fixed in 1ml PFA for 6 hours and then transferred to 70% ethanol. Tails of offspring of *Lepr*$^{db/+}$ dams were collected and frozen for genotyping (see **Section 4.2.4.3**).

4.4.4.6.2 PN21 tissue collection

All *Lepr*$^{db/+}$ and +/+ dams littered down on E19.5. At PN2, pup sex and body weights were recorded, and pups were weighed approximately every second or third day thereafter. Pups of *Lepr*$^{db/+}$ dams were identified from PN9 by paw pad/toe tattoo and a 1mm tail sample was taken to genotype litters as previously described (**Section 4.2.4.3**). At PN21, pups were weighed and one to two pups of each sex, with body weights closest to the litter mean, were collected. Only +/+ pups were collected.

Tissue of PN21 offspring was collected as described previously (**Section 3.4.1.2**). Briefly, pups were anaesthetised and blood was collected. Offspring were perfusion fixed with Karnovsky's fixative. Following fixation, the left and right kidneys were carefully excised, weighed and placed in formalin.

4.4.4.7 Assessment of offspring nephron number

Kidneys of E18.5 embryos and PN21 pups from *Lepr*$^{db/+}$ and +/+ dams were analysed for total nephron number using a modified version of the physical disector/fractionator method as described in **Section 3.4.5**.

Briefly, total glomerular number was determined using the physical disector/fractionator combination, an unbiased stereological method. Fixed kidneys were processed to paraffin and exhaustively

sectioned at 4 µm (E18.5) or 5 µm (PN21) using a microtome. 10 evenly spaced section pairs were systematically sampled and histochemically stained with PNA to localise the plasma membrane of glomerular podocytes. Sections were counterstained with Haematoxylin. Section pairs were used to count PNA-positive glomeruli using the physical disector/fractionator combination. Kidney volume at E18.5 and PN21, and mean glomerular volume and total glomerular volume at PN21 were estimated.

4.4.4.8 Statistical analysis

Data were analysed using SPSS software and GraphPad Prism software. Maternal weights, food intake and plasma insulin and leptin were analysed by independent samples t-test. GTTs were analysed by repeated measures ANOVA. A glucose AUC value was calculated using GraphPad and analysed by independent samples t-test. Measures of offspring growth were analysed by two-way ANOVA with maternal genotype and offspring sex as main effects, and incorporating a mixed linear model to account for any intra-litter bias (Festing, 2006). Measures of offspring plasma content and kidney development were analysed by two-way ANOVA with maternal genotype and offspring sex as main effects. Spearman's rank co-efficient with Bonferroni correction was used to measure associations between kidney development and independent variables of interest (offspring body weight, offspring kidney volume, maternal glucose AUC and maternal weight). Spearman's rank coefficient and multiple regression analysis were performed using STATA software. Throughout *n* refers to the number of dams or litters. Offspring sex was incorporated as a variable in all analyses but had no statistically significant effect unless otherwise stated. Data were tested for normality using a D'Agostino-Pearson omnibus normality test. Data are presented as mean ± SEM. $P<0.05$ was considered statistically significant.

4.4.5 CHAPTER 4C: Results - Characterisation of *Lepr*$^{db/+}$ mice as a model of gestational diabetes

Based on preliminary analyses outlined in sub-chapters 4A and 4B, a full-scale study of *Lepr*$^{db/+}$ mice as a model of gestational diabetes was carried out, with the recognition that this mouse line does not produce a consistent phenotype of impaired maternal glucose tolerance at E17.5. These results are presented below. Note that the small cohort of +/+ and *Lepr*$^{db/+}$ mice presented in chapter 4B are included in the data presented below.

4.4.5.1 *Lepr*$^{db/+}$ mice exhibit pre-gestational glucose intolerance

To assess glucose profiles prior to pregnancy, +/+ and *Lepr*$^{db/+}$ mice were subject to a GTT before mating (**Figure 4.8**). Compared with +/+ mice, there was a trend for *Lepr*$^{db/+}$ mice to be glucose intolerant before pregnancy (P=0.057). When assessed as glucose AUC, the glucose profile of *Lepr*$^{db/+}$ mice was significantly elevated (+/+ 925.7 ± 32.6 mmol/l.min, *Lepr*$^{db/+}$ 1007.1 ± 20.9 mmol/l.min; P=0.037), indicating that *Lepr*$^{db/+}$ mice constitute a model of pre-gestational glucose intolerance.

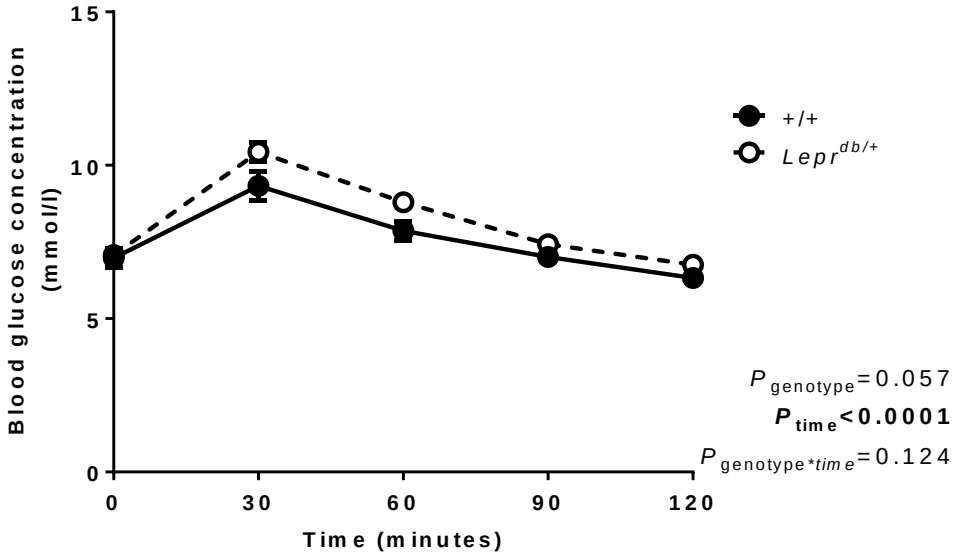


Figure 4.8 Pre-pregnancy glucose tolerance tests
GTT analysed by repeated measures ANOVA for maternal genotype and time. Values are mean ± SEM. n(+/+)=17, n(*Lepr*$^{db/+}$)=38.

4.4.5.2 Incomplete penetrance of glucose intolerance in *Lepr*$^{db/+}$ dams at E17.5 and selection criteria

Ongoing assessment of late gestation GTTs continued to show variable penetrance in *Lepr*$^{db/+}$ dams. In order to identify *Lepr*$^{db/+}$ mice with 'normal glucose tolerance' or 'impaired glucose tolerance', a glucose AUC was calculated for each dam based on the E17.5 GTT. *Lepr*$^{db/+}$ mice with a glucose AUC

greater than one standard deviation above the mean AUC value for +/+ mice were classified as 'impaired glucose tolerant' (IGT *Lepr*$^{db/+}$). *Lepr*$^{db/+}$ mice with a glucose AUC less than one standard deviation above the mean AUC value for +/+ mice were classified as 'normal glucose tolerant' (NGT *Lepr*$^{db/+}$). Approximately 45% of *Lepr*$^{db/+}$ mice displayed an elevated glucose profile (AUC > 877 mmol/l.min at E17.5). 14% of +/+ mice presented with an E17.5 glucose AUC > 877 mmol/l.min and were excluded from analysis. Data presented below represents these selected +/+ and IGT *Lepr*$^{db/+}$ dams, unless otherwise stated.

4.4.5.3 IGT *Lepr*$^{db/+}$ dams have elevated glucose profiles prior to pregnancy and at E12.5 and E17.5

Following the selection of impaired glucose tolerant *Lepr*$^{db/+}$ mice in **Section 4.4.5.2**, pre-pregnancy glucose tolerance was re-assessed in +/+ and IGT *Lepr*$^{db/+}$ mice fitting the selection criteria. Compared with +/+ mice, IGT *Lepr*$^{db/+}$ mice (AUC > 877 mmol/l.min at E17.5) were found to be significantly glucose intolerant prior to pregnancy (P=0.003; **Figure 4.9A**). Post-hoc analysis identified elevated blood glucose concentrations at 30 minutes (P=0.002), 60 minutes (P=0.002) and 120 minutes (P=0.028) after a glucose load, with trends at 90 minutes (P=0.06) and at fasting (P=0.066). Compared with +/+ mice, IGT *Lepr*$^{db/+}$ mice had an 18% increase in glucose AUC values before pregnancy (+/+ 873.9 ± 13.5 mmol/l.min, IGT *Lepr*$^{db/+}$ 1028.0 ± 23.5 mmol/l.min; P<0.0001).

Glucose intolerance was maintained in IGT *Lepr*$^{db/+}$ dams at E12.5 (P=0.002; **Figure 4.9B**). This corresponds to the gestational age at which nephrogenesis commences in offspring. Compared with +/+ dams, IGT *Lepr*$^{db/+}$ dams had elevated glucose levels at 30 minutes (P=0.004), 60 minutes (P=0.027) and 90 minutes (P=0.005) after a glucose load, and had mild fasting hyperglycaemia (P=0.019). As seen prior to pregnancy, an 18% increase in glucose AUC was observed in mid-gestation in IGT *Lepr*$^{db/+}$ dams (+/+ 833.7 ± 32.9 mmol/l.min, IGT *Lepr*$^{db/+}$ 983.2 ± 26.0 mmol/l.min; P=0.002).

As expected, selected IGT *Lepr*$^{db/+}$ mice exhibited glucose intolerance at E17.5 (P<0.0001; **Figure 4.9C**). Post-hoc analysis revealed a significantly elevated fasting blood glucose concentration in IGT *Lepr*$^{db/+}$ dams (P<0.0001), as well as an elevated glucose profile at all time points following a glucose load (P<0.0001 at 30, 60, 90 and 120 minutes). Glucose AUC was 36% greater at E17.5 in IGT *Lepr*$^{db/+}$ mice compared with +/+ mice (+/+ 735.6 ± 22.9 mmol/l.min, IGT *Lepr*$^{db/+}$ 1002.9 ± 25.2 mmol/l.min; P<0.0001).

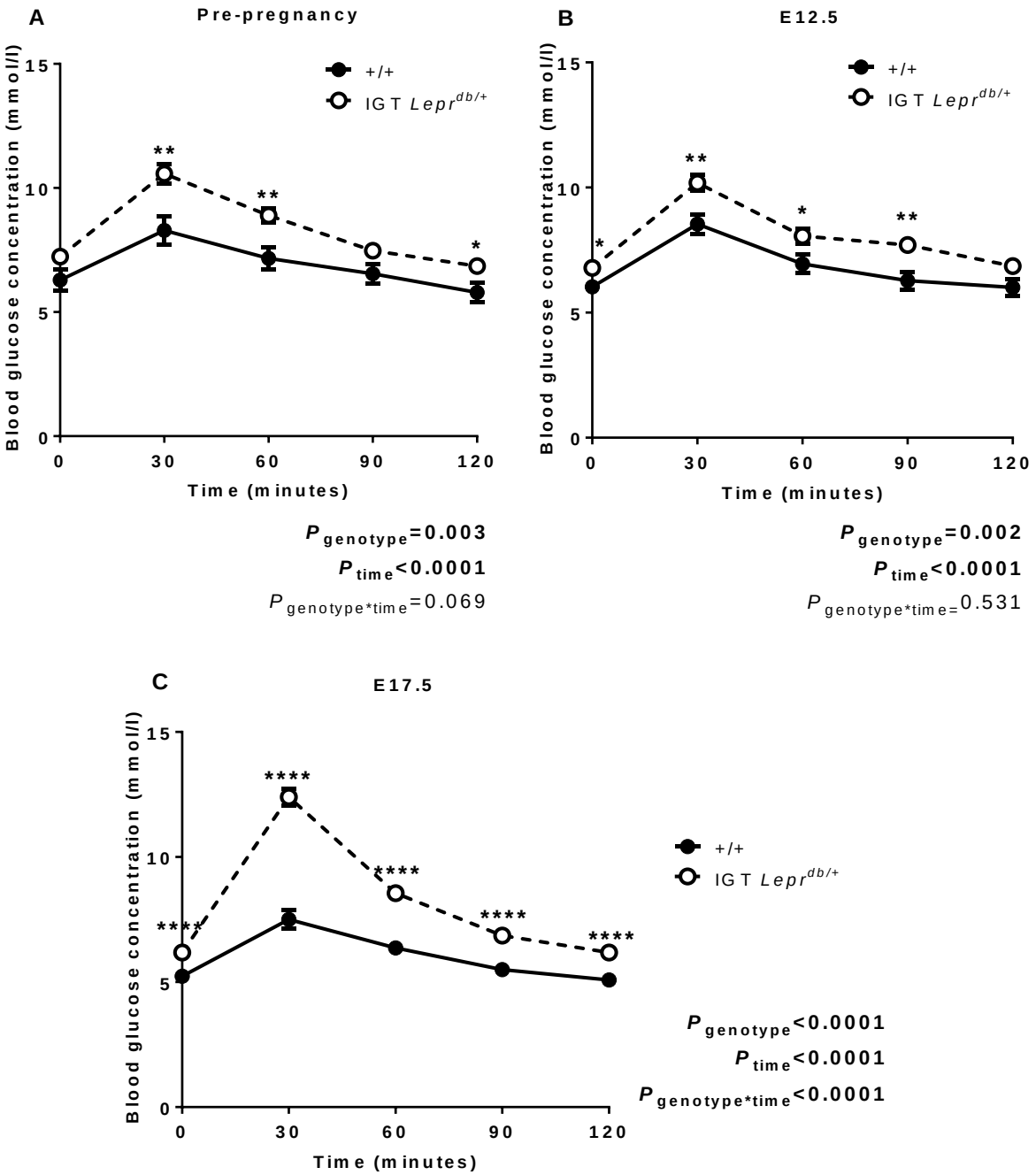


Figure 4.9 Pre-pregnancy and pregnancy glucose tolerance tests

A Pre-pregnancy, **B** E12.5 and **C** E17.5 GTT curves in selected +/+ and IGT *Lepr*$^{db/+}$ mice. Data analysed by repeated measures ANOVA for maternal genotype and time. Post-hoc analysis by independent samples t-test. Values are mean ± SEM. *n*(+/+ pre-pregnancy)=13, *n*(IGT *Lepr*$^{db/+}$ pre-pregnancy)=28. *n*(+/+ E12.5)=10, *n*(IGT *Lepr*$^{db/+}$ E12.5)=16. *n*(+/+ E17.5)=37, *n*(IGT *Lepr*$^{db/+}$ E17.5)=51. **P*<0.05, ***P*<0.01, *****P*<0.00001 refer to differences between the two groups at the each time point.

It should be noted that there was no overall difference in glucose tolerance curves in IGT *Lepr*$^{db/+}$ mice before pregnancy and in late pregnancy (E17.5) (*P*=0.278, **Figure 4.10**). However, during late pregnancy IGT *Lepr*$^{db/+}$ mice had a statistically significant greater mean blood glucose concentration at 30 minutes yet reduced glucose levels at 0, 90 and 120 minutes compared with pre-pregnancy glucose levels. In

+/+ mice, glucose tolerance curves were lower at all time points of the E17.5 GTT than prior to pregnancy (P<0.0001; **Figure 4.11**). Analysis of glucose AUC values by repeated measures ANOVA further supports this: glucose AUC levels remained constant in IGT *Lepr*$^{db/+}$ mice from the non-pregnant to pregnant state yet decreased by 12% in pregnant +/+ mice (+/+ pre-pregnancy 873.9 ± 29.7 mmol/l.min, E17.5 768.9 ± 28.3 mmol/l.min; IGT *Lepr*$^{db/+}$ pre-pregnancy 1028.0 ± 20.2 mmol/l.min, E17.5 1031.9 ± 19.3 mmol/l.min; $P_{gravida}$=0.011, $P_{genotype}$<0.0001 **Figure 4.12**).

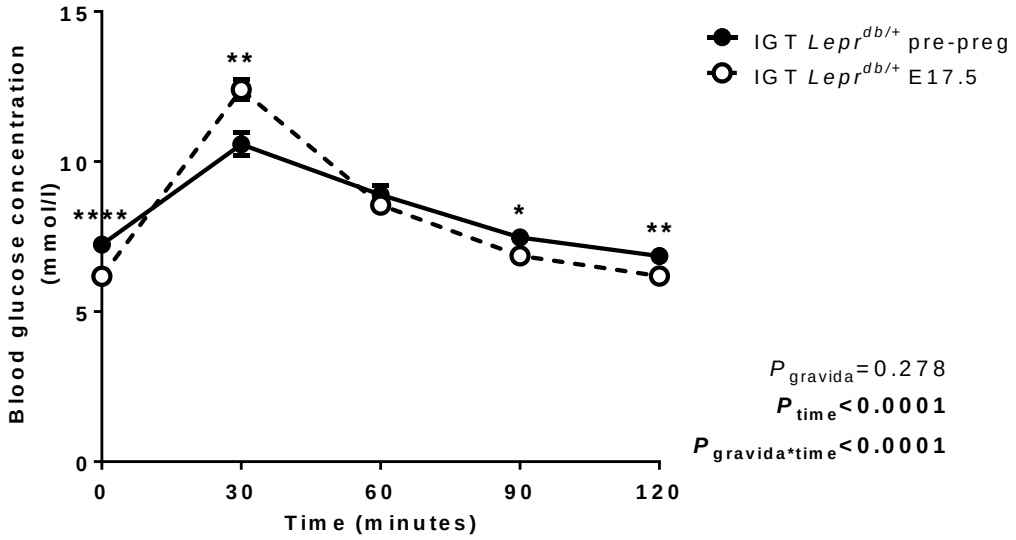


Figure 4.10 Glucose tolerance tests in IGT *Lepr*$^{db/+}$ mice prior to pregnancy and at E17.5
GTT analysed by repeated measures ANOVA for gravida and time. Post-hoc analysis by independent samples t-test. Values are mean ± SEM. n(pre-pregnancy)=32, n(E17.5)=51. *P<0.05, **P<0.01, and ****P<0.0001 refer to the difference between the two groups at each time point.

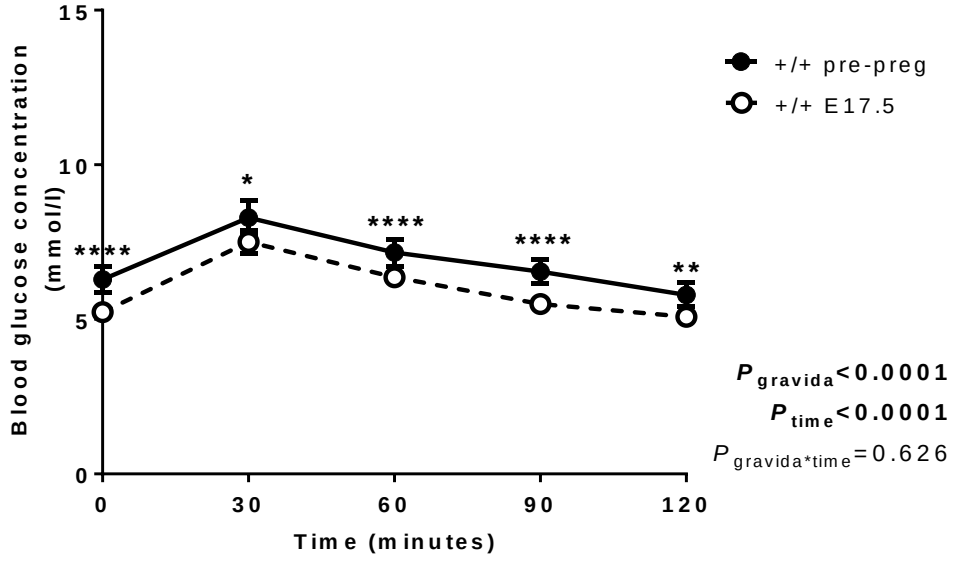


Figure 4.11 Glucose tolerance tests in +/+ mice prior to pregnancy and at E17.5
GTT analysed by repeated measures ANOVA for gravida and time. Post-hoc analysis by independent samples t-test. Values are mean ± SEM. *n*(pre-pregnancy)=13, *n*(E17.5)=37. **P*<0.05, ***P*<0.01, and *****P*<0.0001 refer to the difference between the two groups at each time point.

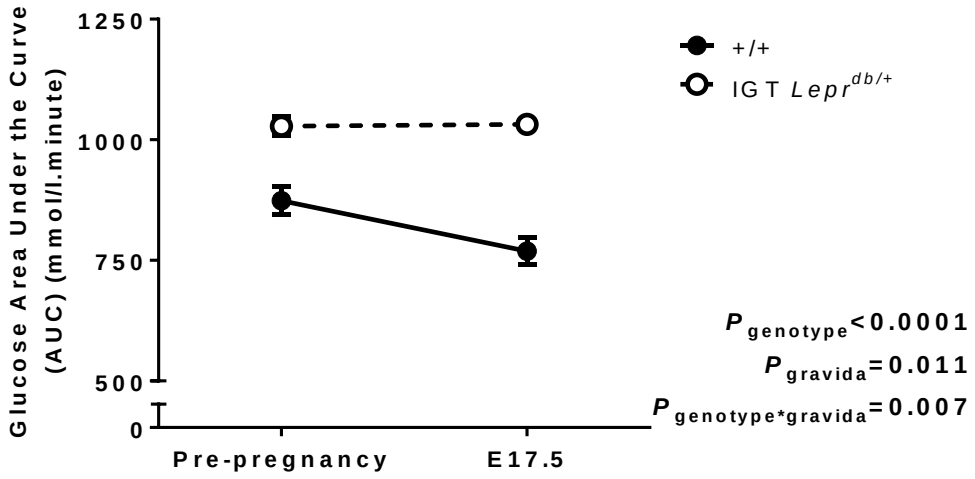


Figure 4.12 Glucose AUC prior to pregnancy and at E17.5
Glucose AUC in +/+ mice and IGT *Lepr*$^{db/+}$ mice prior to pregnancy and at E17.5. Analysis by repeated measures ANOVA for gravida and genotype. Values are mean ± SEM. *n*(+/+)=13, *n*(IGT *Lepr*$^{db/+}$)=28.

4.4.5.4 IGT *Lepr*$^{db/+}$ dams have increased weight gain and food intake in pregnancy

Compared with +/+ dams, IGT *Lepr*$^{db/+}$ mice were heavier at E0.5 (*P*<0.0001) and E17.5 (*P*<0.0001) (**Table 4.6**). Weight gain and food intake, measured between E0.5 and E17.5, were 18% greater in IGT

Lepr$^{db/+}$ dams than +/+ dams (*P*=0.021 and *P*=0.005, respectively). The greater weight gain observed in IGT *Lepr*$^{db/+}$ dams may be associated with a greater litter size (to be described in **Section 4.4.6.2.1**).

Table 4.6 Maternal body weight, weight gain and food intake

	+/+ (*n*)	**IGT *Lepr*$^{db/+}$ (*n*)**	***P***
Body weight E0.5, g	19.7 ± 0.6 (35)	23.0 ± 0.6 (51)	**<0.0001**
Body weight E17.5, g	31.6 ± 1.0 (35)	37.3 ± 0.5 (51)	**<0.0001**
Pregnancy weight gain, g	12.3 ± 0.3 (35)	13.9 ± 0.3 (51)	**0.021**
Food intake, g	38.7 ± 1.6 (8)	46.0 ± 1.5 (13)	**0.005**

Analysis by independent samples t-test. Values are mean ± SEM.

4.4.5.5 IGT *Lepr*$^{db/+}$ mice exhibit hyperinsulinaemia and hyperleptinaemia in late gestation

Maternal plasma insulin and leptin were quantified at E17.5. IGT *Lepr*$^{db/+}$ dams had significantly elevated insulin (+/+ 0.30 ± 0.04 ng/ml, IGT *Lepr*$^{db/+}$ 1.09 ± 0.24 ng/ml; *P*=0.004; **Figure 4.13A**) and leptin levels (+/+ 9.06 ± 0.60 ng/ml, IGT *Lepr*$^{db/+}$ 17.74 ± 1.24 ng/ml; *P*<0.0001; **Figure 4.13B**) compared with +/+ dams. This represents an almost 4-fold increase in insulin and a 2-fold increase in leptin.

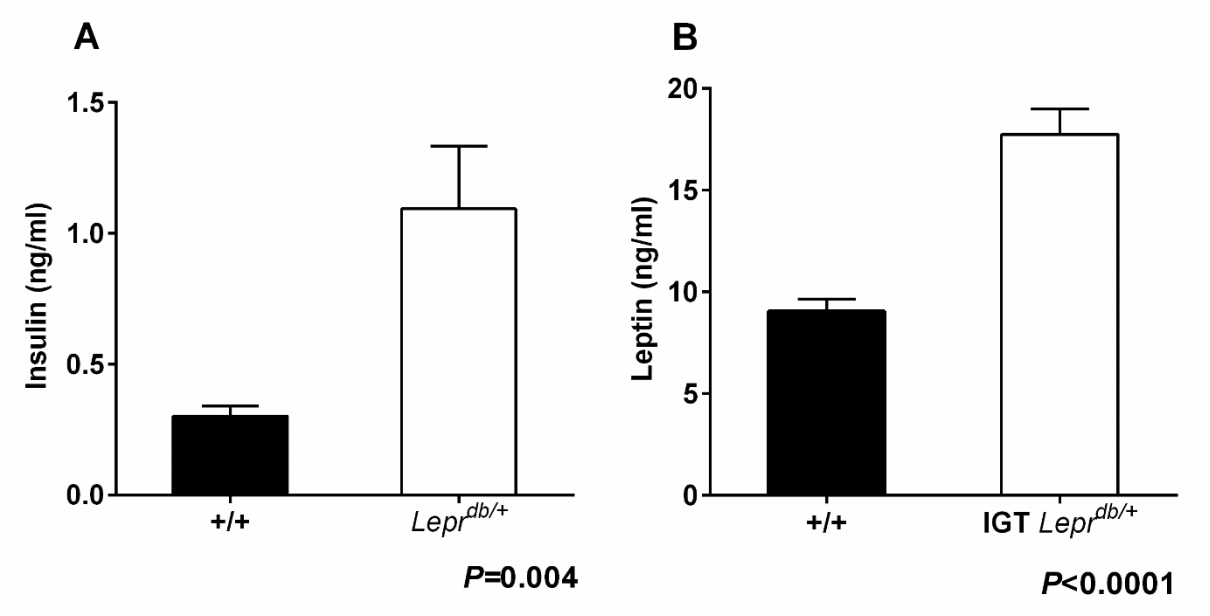


Figure 4.13 Maternal insulin and leptin levels at E17.5

Mean maternal plasma **A** insulin and **B** leptin levels at E17.5 in +/+ dams and IGT *Lepr*$^{db/+}$ dams. Plasma content analysed by independent samples t-test. Values are mean ± SEM. *n*(+/+)=12, *n*(IGT *Lepr*$^{db/+}$)=12.

4.4.5.6 Maternal body weight correlates with glucose intolerance in late gestation

Associations between glucose intolerance in late gestation and maternal variables are presented in **Table 4.7**. Maternal glucose AUC at E17.5 correlated significantly with maternal weight at conception,

weight at E17.5 and weight gain in pregnancy. There was no association between glucose AUC and insulin levels, however 30 minute blood glucose levels did correlate positively with maternal insulin.

Table 4.7 Spearman's rank coefficients for associations between maternal glucose intolerance in late gestation and independent variables

		Glucose AUC	Fasting glucose E17.5	30 min. glucose E17.5	Weight E0.5	Weight E17.5	Weight gain
Fasting glucose E17.5	**rho**	0.112					
	P	0.603					
30 minute glucose E17.5	**rho**	**0.857**	0.118				
	P	**<0.0001**	0.584				
Weight E0.5	**rho**	**0.454**	0.182	**0.598**			
	P	**0.04**	0.430	**0.004**			
Weight E17.5	**rho**	**0.685**	0.082	**0.773**	**0.920**		
	P	**0.040**	0.704	**<0.0001**	**<0.0001**		
Weight gain	**rho**	**0.723**	0.028	**0.733**	**0.635**	**0.861**	
	P	**0.0002**	0.904	**0.0002**	**0.002**	**<0.0001**	
Insulin E18.5	**rho**	0.328	0.160	**0.491**	**0.675**	**0.628**	0.312
	P	0.118	0.454	**0.015**	**0.0008**	**0.001**	0.169

Spearman's rho (top value), *P* value (bottom value). Values in bold are statistically significant.

4.4.5.7 Maternal weight is the only predictor of impaired glucose tolerance

Less than half of all tested *Lepr*$^{db/+}$ dams were found to exhibit impaired glucose tolerance in pregnancy (as determined by the criteria described in **Section 4.4.5.2**). To determine if weight, weight gain, age, pre-pregnancy glucose AUC and/or food intake predicted glucose intolerance, analyses were performed between dams that were glucose intolerant at E17.5 and those that were not. Note: Initially, any *Lepr*$^{db/+}$ dam with a glucose concentration less than 10 mmol/l 30 minutes after a glucose load was deemed as having NGT. These mice were culled before the completion of the GTT and a glucose AUC could not be calculated. Later in the study it was decided that glucose AUC would be a more accurate method to define glucose tolerance, rather than a peak glucose level greater than 10 mmol/l 30 minutes after a glucose load, and all time points of a GTT were performed hereafter. Because of this, the number of 'NGT' mice presented are fewer than what is expected given a penetrance of approximately 45%.

Lepr$^{db/+}$ dams with a glucose AUC > 877 mmol/l.min at E17.5 were defined as IGT and those with a glucose AUC < 877 mmol/l.min at E17.5 were defined as NGT, as previously described (**Section 4.4.5.2**). IGT *Lepr*$^{db/+}$ dams were heavier by approximately 1 g at E0.5 and by 2 g at E17.5 when compared with NGT *Lepr*$^{db/+}$ dams (*P*=0.041 and *P*=0.027, respectively; **Table 4.8**). Pregnancy weight gain and age at

conception did not differ between NGT *Lepr*$^{db/+}$ and IGT *Lepr*$^{db/+}$ dams (**Table 4.8**) and therefore did not predict maternal glucose intolerance.

There was no significant difference in pre-pregnancy glucose AUC between *Lepr*$^{db/+}$ mice with or without impaired glucose tolerance in late gestation (**Table 4.8**). Pre-pregnancy glucose AUC was also compared with +/+ dams by one-way ANOVA (**Figure 4.14**). Pre-pregnancy glucose AUC of NGT *Lepr*$^{db/+}$ mice was similar to both +/+ (P=0.250) and IGT *Lepr*$^{db/+}$ mice (P=0.072) (+/+ 873.9 ± 29.7 mmol/l.min, NGT *Lepr*$^{db/+}$ 937.1 ± 52.8 mmol/l.min, IGT *Lepr*$^{db/+}$ 1028.0 ± 23.5 mmol/l.min).

Food intake during pregnancy was measured in 15 *Lepr*$^{db/+}$ mice and four were identified with NGT. There was no difference in food intake between *Lepr*$^{db/+}$ dams with or without glucose intolerance (**Table 4.8**). Interestingly, when compared with food intake in +/+ dams (+/+ 38.2 ± 1.5 g, n=8), *Lepr*$^{db/+}$ dams consumed significantly more food during pregnancy regardless of glucose tolerance (**Figure 4.15**).

Table 4.8 Maternal body weight, weight gain, age, pre-pregnancy glucose AUC and food intake in NGT and IGT *Lepr*$^{db/+}$ dams

	NGT *Lepr*$^{db/+}$ (n)	**IGT *Lepr*$^{db/+}$** (n)	***P***
Body weight E0.5, g	22.3 ± 0.4 (21)	23.5 ± 0.3 (48)	**0.041**
Body weight E17.5, g	35.4 ± 0.7 (21)	37.3 ± 0.5 (48)	**0.027**
Pregnancy weight gain, g	13.2 ± 0.4 (21)	13.9 ± 0.3 (48)	0.109
Age at E0.5, days	85.1 ± 5.8 (21)	88.4 ± 3.5 (48)	0.613
Pre-pregnancy glucose AUC, mmol/l.min	937.1 ± 52.8 (7)	1028.0 ± 23.5 (28)	0.159
Food intake, g	46.2 ± 0.6 (4)	45.3 ± 1.6 (11)	0.749

Analysis by independent samples t-test. Values are mean ± SEM.

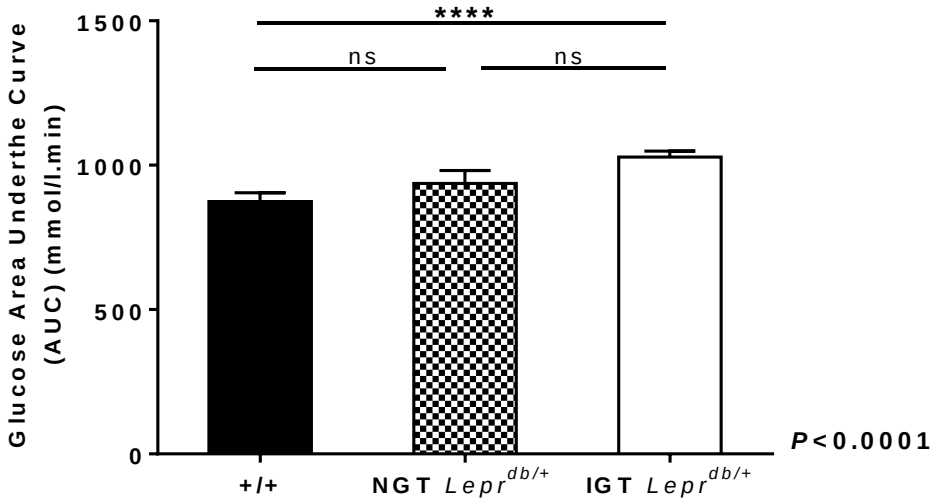


Figure 4.14 Pre-pregnancy glucose AUC in +/+, NGT *Lepr*$^{db/+}$ and IGT *Lepr*$^{db/+}$ mice
Data analysed by one-way ANOVA. Post-hoc analysis by Fisher's least significant difference. Values are mean ± SEM. *n*(+/+)=13, *n*(NGT *Lepr*$^{db/+}$)=7, (IGT *Lepr*$^{db/+}$)=28. ****P<0.0001.

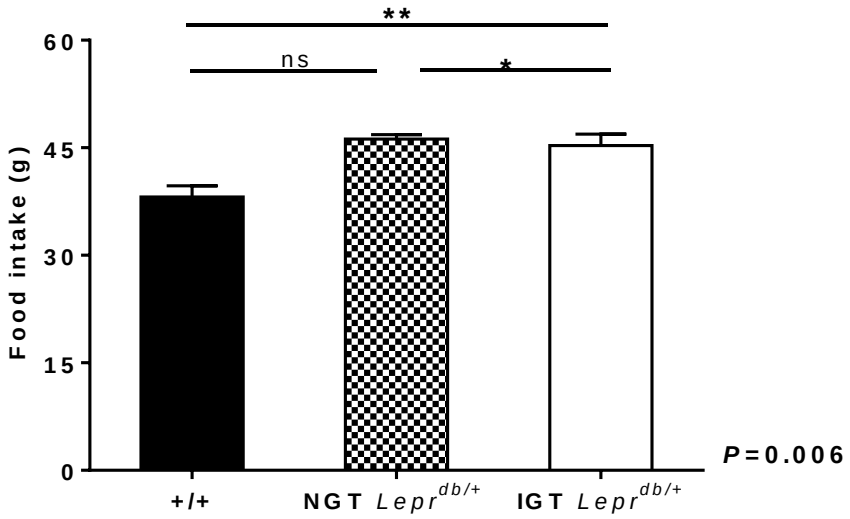


Figure 4.15 Food intake in pregnancy in +/+, NGT *Lepr*$^{db/+}$ and IGT *Lepr*$^{db/+}$ mice
Data analysed by one-way ANOVA. Post-hoc analysis by Fisher's least significant difference. Values are mean ± SEM. *n*(+/+)=8, *n*(NGT *Lepr*$^{db/+}$)=4, (IGT *Lepr*$^{db/+}$)=11. *P<0.05, **P<0.01.

4.4.5.8 Maternal glucose tolerance normalises in IGT *Lepr*$^{db/+}$ dams after pregnancy

Mice underwent GTTs in the postnatal period at PN4 and PN21. Due to the stressful circumstances that repeat GTTs impose on the dam and litter, glucose handling was assessed in only a small cohort of +/+ dams (*n*=7) and IGT *Lepr*$^{db/+}$ dams (*n*=8).

GTTs performed before pregnancy, during pregnancy at E17.5 and postnatally at PN4 and PN21 were converted to glucose AUC values and analysed by repeated measures ANOVA. Overall, IGT *Lepr*$^{db/+}$ dams had significantly elevated glucose profiles compared with +/+ dams (*P*=0.004; **Figure 4.16**). Parallel to what was reported in the full cohort of IGT *Lepr*$^{db/+}$ dams in **Section 4.4.5.3**, IGT *Lepr*$^{db/+}$ dams analysed here exhibited glucose intolerance prior to pregnancy (*P*=0.009) and at E17.5 (*P*<0.0001). However in the postnatal period glucose AUC values were similar to +/+ dams at PN4 (*P*=0.460) and PN21 (*P*=0.794).

Body weight was also monitored across the postnatal period. Overall, IGT *Lepr*$^{db/+}$ dams were significantly heavier over the period analysed (from before pregnancy to PN21) (*P*=0.013; **Figure 4.17**). Post-hoc analysis revealed statistically elevated body weights in IGT *Lepr*$^{db/+}$ dams before pregnancy (*P*=0.032), at PN4 (*P*=0.010) and at PN21 (*P*=0.044). There was no statistical difference in body weight between +/+ and IGT *Lepr*$^{db/+}$ dams at E17.5. This may be due to the low number of dams studied in the postnatal period as an 18% increase in late gestational body weight was reported in the full cohort of mice in **Section 4.4.5.4**.

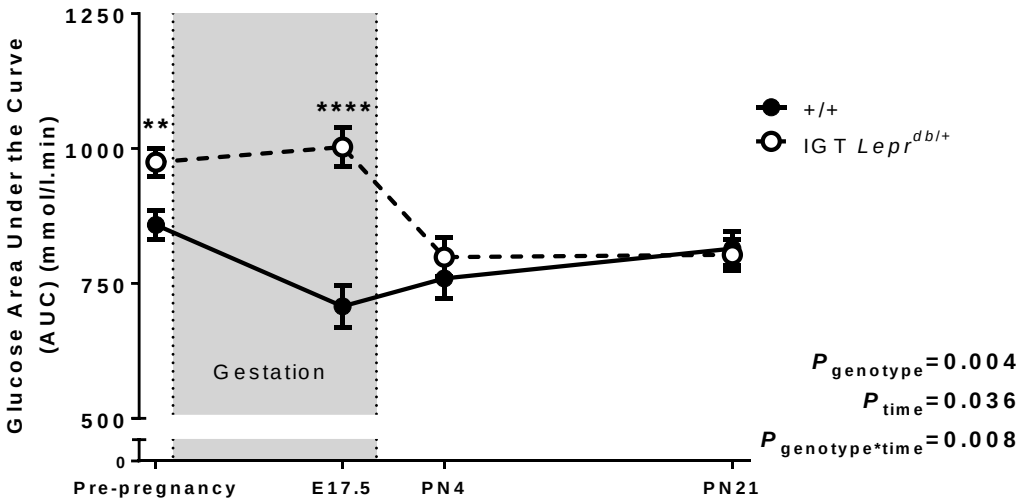


Figure 4.16 Postnatal glucose AUC

Postnatal glucose AUC of +/+ dams and IGT *Lepr*$^{db/+}$ dams from before pregnancy up to PN21. Data analysed by repeated measures ANOVA. Post-hoc analysis by independent samples t-test. Values are mean ± SEM. *n*(+/+)=7, *n*(IGT *Lepr*$^{db/+}$)=8. ****P*<0.01 and *****P*<0.0001 refer to the difference between the two groups at each time point.

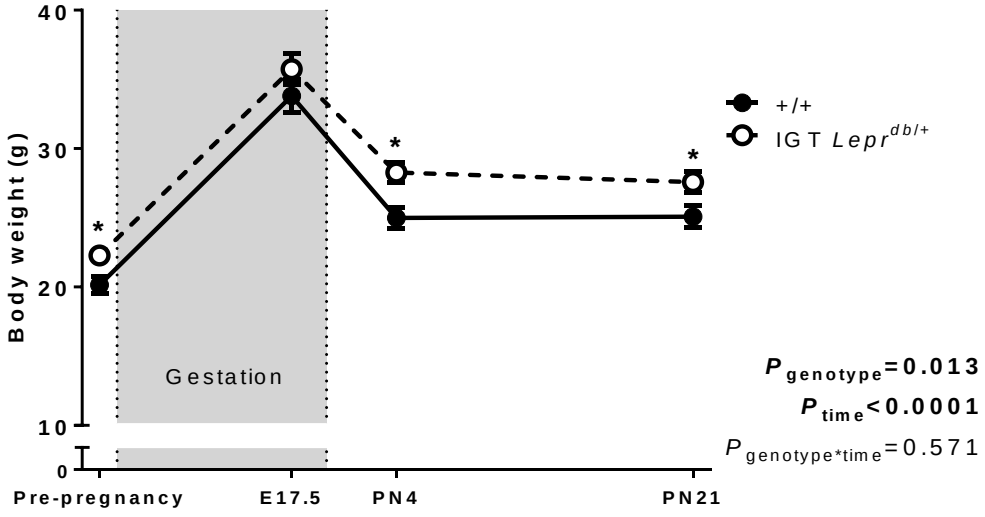


Figure 4.17 Postnatal body weight

Postnatal weight of +/+ dams and IGT *Lepr*$^{db/+}$ dams between PN4 and PN21. Data analysed by repeated measures ANOVA. Post-hoc analysis by independent samples t-test. Values are mean ± SEM. *n*(+/+)=7, *n*(IGT *Lepr*$^{db/+}$)=8. **P*<0.05 refers to the difference between the two groups at each time point.

4.4.6 CHAPTER 4C: Results – Growth parameters and kidney development in offspring of *Lepr*$^{db/+}$ dams

Offspring of +/+ and *Lepr*$^{db/+}$ dams were collected at two time points to analyse kidney development: E18.5 and PN21.

4.4.6.1 Offspring growth and litter characteristics at E18.5

4.4.6.1.1 Litter size is similar

Litter size was comparable between dams with normal and impaired glucose tolerance (+/+ 5.7 ± 0.4 embryos, IGT *Lepr*$^{db/+}$ 6.6 ± 0.5 embryos; *P*=0.207). The proportion of alive and resorbed embryos was also similar in the two groups; 87% of embryos collected from +/+ were viable and 88% of embryos from IGT *Lepr*$^{db/+}$ dams were viable (**Figure 4.18**). One dead embryo was observed in one IGT *Lepr*$^{db/+}$ litter. Data were analysed using the chi-squared statistic (2x3 contingency table) which did not show a significant difference between the two groups (*P*=0.685).

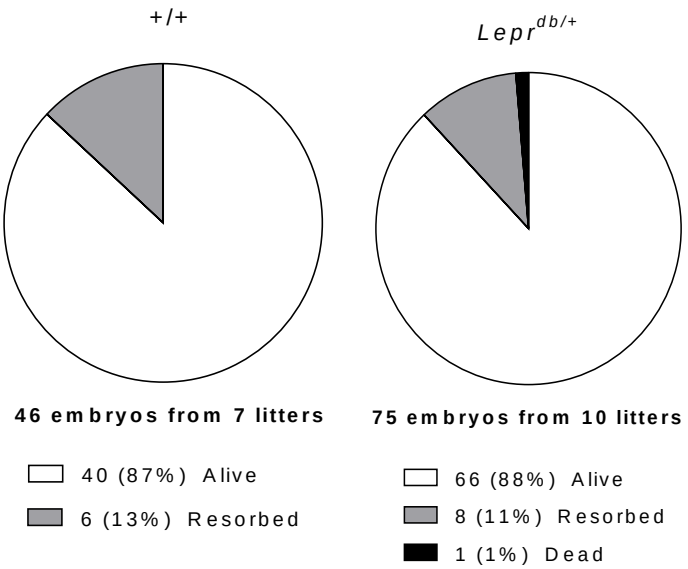


Figure 4.18 Litter characteristics at E18.5
Proportion of alive, resorbed and dead embryos in +/+ litters and IGT *Lepr*$^{db/+}$ litters.

4.4.6.1.2 Normal uterus weight

There was no statistical difference in uterus weight between +/+ and IGT *Lepr*$^{db/+}$ dams (+/+ 9.1 ± 0.7 g, IGT *Lepr*$^{db/+}$ 9.8 ± 0.6 g; *P*=0.481). Pregnancy weight gain minus gravid uterus weight was higher in IGT *Lepr*$^{db/+}$ dams (+/+ 3.6 ± 0.3 g, IGT *Lepr*$^{db/+}$ 4.9 ± 1.4 g; *P*=0.026).

4.4.6.1.3 Offspring of IGT *Lepr*$^{db/+}$ dams are not macrosomic but have larger placentae

Only wild type +/+ offspring of +/+ and IGT *Lepr*$^{db/+}$ dams were analysed. Embryos of IGT *Lepr*$^{db/+}$ dams were not macrosomic, with no effect of maternal glucose intolerance on offspring body weight at E18.5

(+/+ 1,054.9 ± 33.0 mg, IGT *Lepr*$^{db/+}$ 1,045.7 ± 21.6 mg; *P*=0.773; **Figure 4.19**). There was no difference in the ratio of male to female offspring (*P*=0.585). Male embryos were significantly heavier than female embryos (*P*=0.003). Placental tissue weight was greater (+/+ 91.7 ± 2.2 mg, IGT *Lepr*$^{db/+}$ 99.1 ± 2.2 mg; *P*=0.038) and the fetal: placental weight ratio was lower (+/+ 11.5 ± 0.2, IGT *Lepr*$^{db/+}$ 10.6 ± 0.2; *P*=0.015) in glucose intolerant dams (**Figure 4.20A** and **Figure 4.20B**, respectively). Placentae from male embryos were heavier than those from female embryos (*P*=0.007). There was no difference in crown rump length (+/+ 21.0 ± 0.3 mm, IGT *Lepr*$^{db/+}$ 21.0 ± 0.2 mm; *P*=0.913), head diameter (+/+ 11.2 ± 0.1 mm, IGT *Lepr*$^{db/+}$ 11.2 ± 0.2 mm; *P*=0.584) or ponderal index (+/+ 0.115 ± 0.002 mg/mm^3, IGT *Lepr*$^{db/+}$ 0.113 ± 0.002 mg/mm^3; *P*=0.573) between groups. There was no difference in the ratio of male to female embryos between the two groups (*P*=0.579).

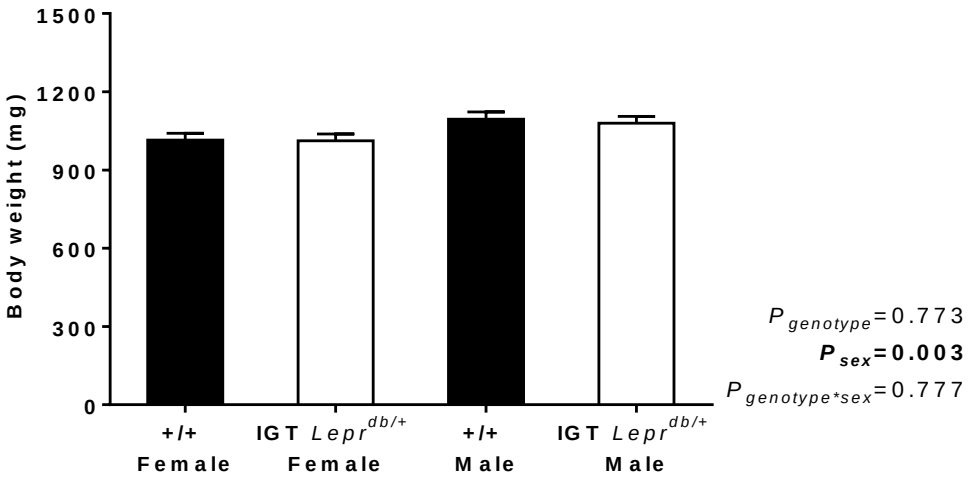


Figure 4.19 Offspring body weight at E18.5

Body weight of female and male embryos of +/+ and IGT *Lepr*$^{db/+}$ dams. Analysis by two-way ANOVA with maternal genotype and sex as variables, weighted for litter using a linear mixed model. Values are mean ± SEM. *n*(+/+ females)=22 embryos from 7 litters, *n*(+/+ males)=18 embryos from 7 litters, *n*(IGT *Lepr*$^{db/+}$ females)=16 embryos from 10 litters, *n*(IGT *Lepr*$^{db/+}$ males)=17 embryos from 9 litters.

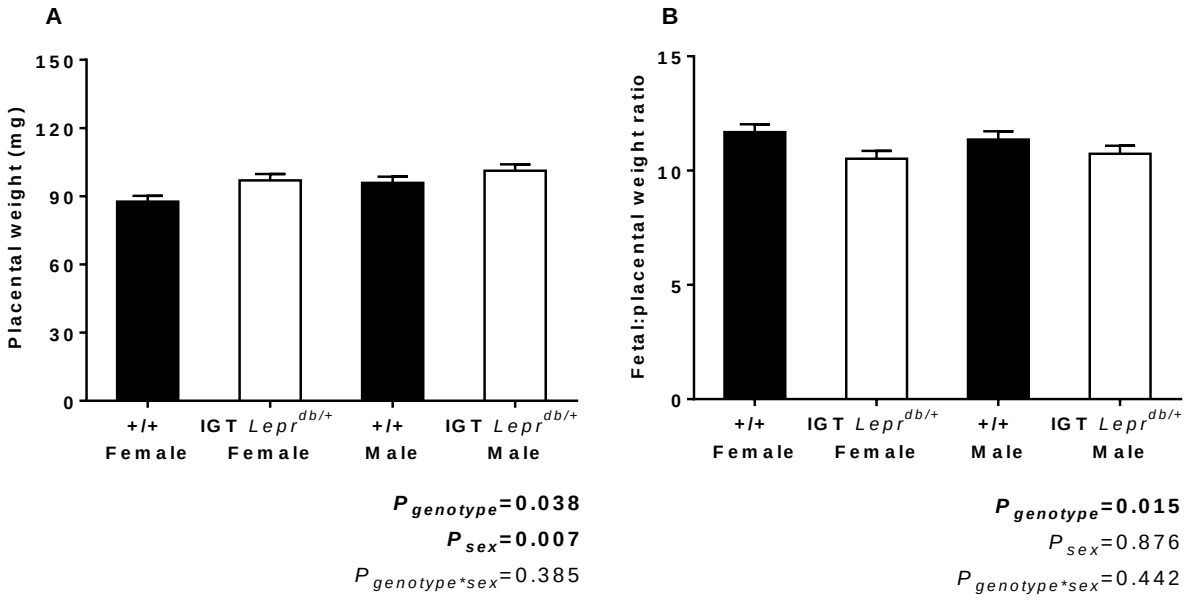


Figure 4.20 Placental weight and fetal: placental weight ratio at E18.5

A Placenta weight and **B** fetal: placental weight ratio of female and male embryos of +/+ and IGT *Lepr*$^{db/+}$ dams. Analysis by two-way ANOVA with maternal genotype and sex as variables, weighted for litter using a linear mixed model. Values are mean ± SEM. *n*(+/+ females)=22 embryos from 7 litters, *n*(+/+ males)=18 embryos from 7 litters, *n*(IGT *Lepr*$^{db/+}$ females)=16 embryos from 10 litters, *n*(IGT *Lepr*$^{db/+}$ males)=17 embryos from 9 litters.

Previous studies report an increase in body weight and macrosomia in offspring born to *Lepr*$^{db/+}$ dams. As shown in **Figure 4.19**, this was not observed in the present study. It is unclear whether previous studies have avoided the confounding effects of genotype by excluding *Lepr*$^{db/+}$ embryos of *Lepr*$^{db/+}$ dams. Consequently, data were analysed incorporating both +/+ and *Lepr*$^{db/+}$ embryos of IGT *Lepr*$^{db/+}$ dams. Again, no difference in body weight was observed (+/+ embryos of +/+ dams 1,055.1 ± 25.1 mg, +/+ and *Lepr*$^{db/+}$ embryos of IGT *Lepr*$^{db/+}$ dams 1,035.1 ± 20.6 mg; *P*=0.549; **Figure 4.21**).

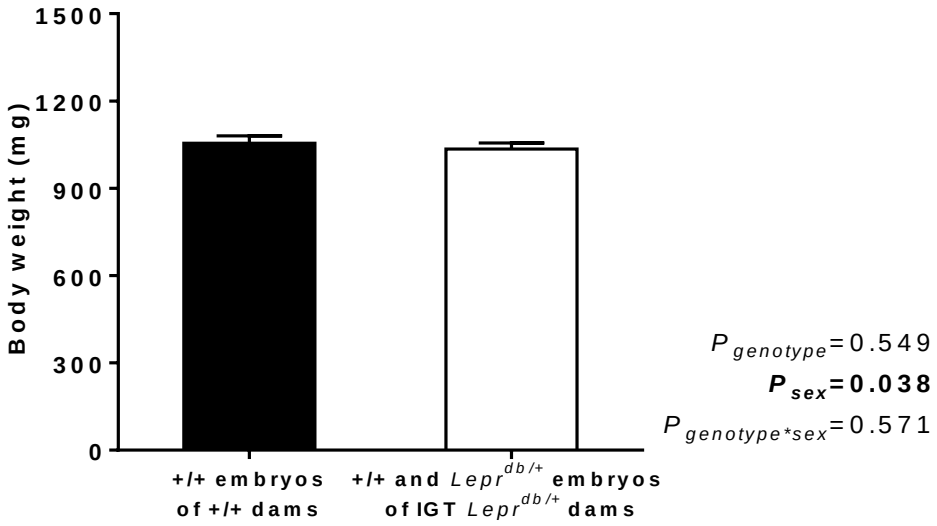


Figure 4.21 Body weight including *Lepr*$^{db/+}$ embryos of IGT *Lepr*$^{db/+}$ dams at E18.5
Body weight of +/+ embryos of +/+ dams, and +/+ and *Lepr*$^{db/+}$ embryos of IGT *Lepr*$^{db/+}$ dams. Analysis by two-way ANOVA with maternal genotype and sex as variables, weighted for litter using a linear mixed model. Values are mean ± SEM. *n*(+/+)=40 embryos from 7 litters, *n*(IGT *Lepr*$^{db/+}$ dam)=63 embryos from 10 litters.

4.4.6.1.4 Offspring of IGT *Lepr*$^{db/+}$ dams are hyperinsulinaemic and hyperleptinaemic

At E17.5, offspring of IGT *Lepr*$^{db/+}$ dams had significantly elevated insulin (+/+ 0.35 ± 0.11 ng/ml, IGT *Lepr*$^{db/+}$ 0.98 ± 0.12 ng/ml; *P*=0.005; **Figure 4.22A**) and leptin levels (+/+ 0.50 ± 0.21 ng/ml, IGT *Lepr*$^{db/+}$ 1.29 ± 0.22 ng/ml; *P*=0.02; **Figure 4.22B**) compared with offspring of +/+ dams.

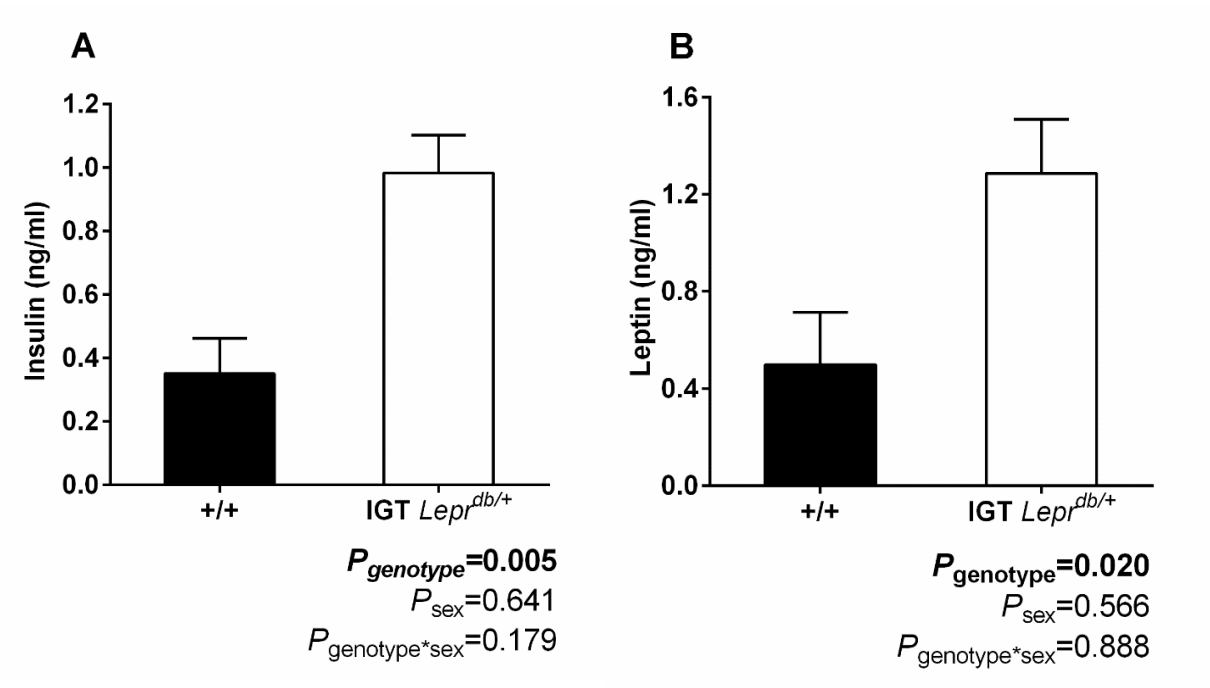


Figure 4.22 Fetal plasma insulin and leptin levels at E17.5

Mean fetal plasma **A** insulin and **B** leptin levels at E17.5 in offspring of +/+ dams and offspring of IGT *Lepr*$^{db/+}$ dams. Plasma content analysed by two-way ANOVA with maternal genotype and sex as variables. Values are mean ± SEM. *n*(+/+)=7 litters, *n*(*Lepr*$^{db/+}$)=6 litters.

4.4.6.2 Offspring growth and litter characteristics at PN21

4.4.6.2.1 Litter size is greater in IGT *Lepr*$^{db/+}$ dams

For mice that comprised the PN21 cohort, litter size was comparable between dams with normal and impaired glucose tolerance (+/+ 5.7 ± 0.3 pups, IGT *Lepr*$^{db/+}$ 6.4 ± 0.3 pups; *P*=0.067). When litter size of E18.5 and PN21 litters were grouped, IGT *Lepr*$^{db/+}$ dams were found to have a statistically greater litter size than +/+ dams (+/+ 5.7 ± 0.2 pups, IGT *Lepr*$^{db/+}$ 6.5 ± 0.2 pups; *P*=0.024; **Figure 4.23**).

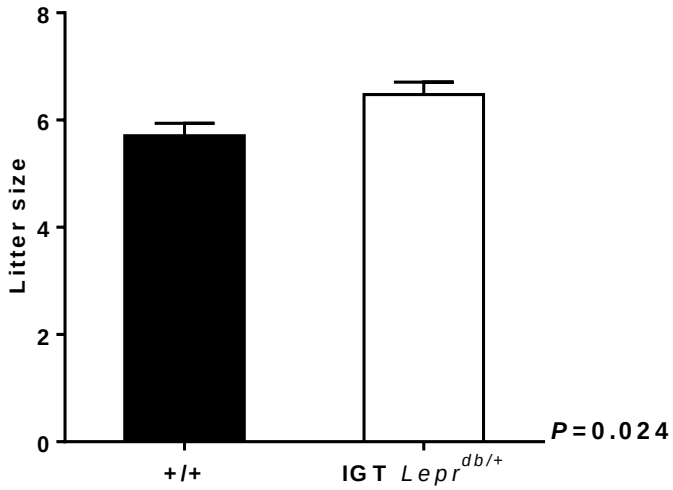


Figure 4.23 Litter size at E18.5 and PN21 (combined)
Litter size in E18.5 and PN21 litters of +/+ and IGT *Lepr*$^{db/+}$ dams. Data analysed by independent samples t-test. Values are mean ± SEM. *n*(+/+)=34 litters, *n*(*Lepr*$^{db/+}$)=40 litters.

4.4.6.2.2 Offspring of IGT *Lepr*$^{db/+}$ dams have normal body weight in the postnatal period

Offspring body weight was recorded between PN2 and PN21. Unfortunately litters were not weighed daily or on specific days and analysis by repeated measures ANOVA was not feasible. Instead, each time point was analysed separately by two-way ANOVA weighted for litter. Body weights of offspring in the early postnatal period (PN2-8) are presented in **Table 4.9**. A greater body weight was observed in pups born to IGT *Lepr*$^{db/+}$ dams at PN2 and PN6. It should be noted that these time points include both +/+ and *Lepr*$^{db/+}$ pups of IGT *Lepr*$^{db/+}$ dams, as genotype was unknown at this time.

Body weights of offspring from PN9-21, once genotypes were determined, are also presented in **Table 4.9**. There was no statistical difference in body weight between offspring of +/+ dams and +/+ offspring of IGT *Lepr*$^{db/+}$ dams between PN9-21. When incorporating offspring of IGT *Lepr*$^{db/+}$ dams with a *Lepr*$^{db/+}$ genotype in the analysis of body weight at PN21, there was a trend for offspring of IGT *Lepr*$^{db/+}$ dams to be heavier than offspring of +/+ dams (*P*=0.065; **Figure 4.24**). Total litter weights were comparable between groups at PN21 (*P*=0.653).

There was a trend for males to be heavier than females at PN9 and PN18. Males were significantly heavier than females at all other time points. There was no difference in the ratio of male to female offspring (*P*=0.111).

Table 4.9 Offspring body weight in the early postnatal period

	Female of +/+	Female of IGT *Lepr*$^{db/+}$	Male of +/+	Male of IGT *Lepr*$^{db/+}$	*n*(+/+) (litters)	*n*(IGT *Lepr*$^{db/+}$) (litters)	P_{diet}	P_{sex}	$P_{diet*sex}$
PN2	1.47 ± 0.05	1.53 ± 0.05	1.53 ± 0.05	1.75 ± 0.05	22	26	**0.006**	**<0.0001**	0.173
PN4	1.96 ± 0.15	2.30 ± 0.10	2.10 ± 0.15	2.44 ± 0.10	9	17	0.067	**0.001**	0.940
PN6	2.84 ± 0.17	3.44 ± 0.14	3.06 ± 0.17	3.58 ± 0.14	9	9	**0.016**	**<0.0001**	0.436
PN8	3.90 ± 0.20	4.46 ± 0.23	4.10 ± 0.20	4.72 ± 0.23	8	6	0.071	**0.001**	0.669
PN9	4.67 ± 0.14	4.94 ± 0.22	4.79 ± 0.14	5.22 ± 0.19	21	11	0.129	0.057	0.428
PN12	6.20 ± 0.16	6.51 ± 0.19	6.33 ± 0.15	6.63 ± 0.18	24	19	0.203	**0.034**	0.913
PN15	6.83 ± 0.17	7.15 ± 0.20	7.08 ± 0.16	7.33 ± 0.18	21	21	0.218	**0.037**	0.735
PN18	7.49 ± 0.22	8.00 ± 0.22	7.67 ± 0.22	8.16 ± 0.20	15	20	0.085	0.069	0.905
PN21	8.40 ± 0.19	8.70 ± 0.22	8.80 ± 0.19	8.93 ± 0.20	25	30	0.323	**0.001**	0.687

Analysis at each postnatal day by two-way ANOVA incorporating maternal diet and sex, weighted for litter using a mixed linear model. Values are mean ± SEM.

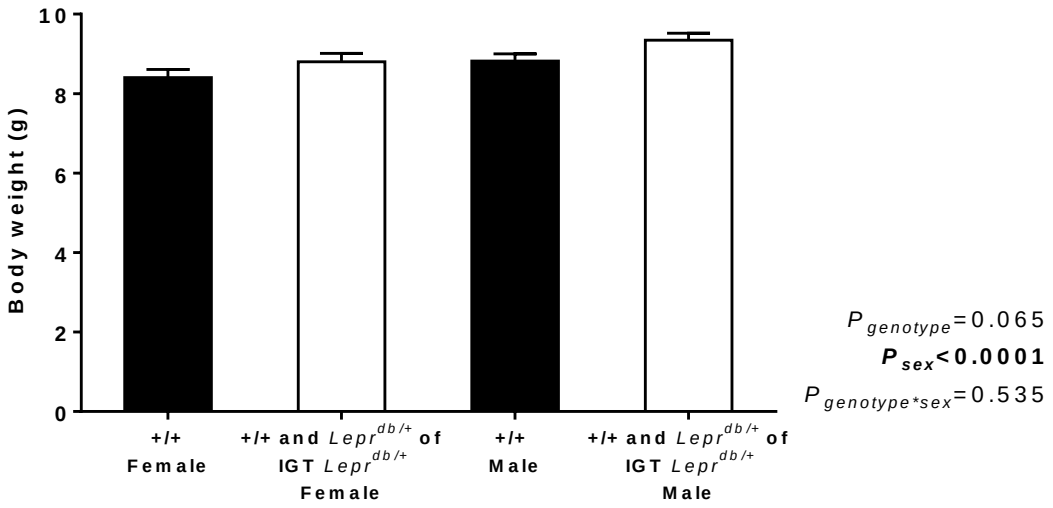


Figure 4.24 Offspring body weight at PN21 incorporating *Lepr*$^{db/+}$ pups of IGT *Lepr*$^{db/+}$ dams
PN21 body weight of pups born to +/+ dams, and +/+ and *Lepr*$^{db/+}$ pups of IGT *Lepr*$^{db/+}$ dams. Analysis by two-way ANOVA with maternal genotype and sex as variables, weighted for litter using a linear mixed model. Values are mean ± SEM. *n*(+/+ female)=77 pups from 25 litters, *n*(+/+ male)=86 pups from 25 litters, *n*(IGT *Lepr*$^{db/+}$ female)=93 pups from 29 litters, *n*(IGT *Lepr*$^{db/+}$ female)=109 pups from 30 litters.

4.4.6.3 Kidney development in offspring of *Lepr*$^{db/+}$ dams with impaired glucose tolerance

4.4.6.3.1 Offspring of IGT *Lepr*$^{db/+}$ dams have a low nephron number at E18.5

Just prior to birth at E18.5, offspring of IGT *Lepr*$^{db/+}$ dams had 17% fewer nephrons than offspring of +/+ dams (+/+ 1684 ± 37 nephrons, IGT *Lepr*$^{db/+}$ 1391 ± 32 nephrons; P<0.0001; **Figure 4.25A**). When expressed relative to body weight, offspring of IGT *Lepr*$^{db/+}$ dams had significantly fewer nephrons per gram of body weight than offspring of +/+ dams (+/+ 1550 ± 40 nephrons/g, IGT *Lepr*$^{db/+}$ 1340 ± 30 nephrons/g; P=0.001, **Figure 4.25B**). Kidney volume in offspring of IGT *Lepr*$^{db/+}$ dams was 12% smaller at E18.5 (+/+ 2.70 ± 0.07 mm^3, IGT *Lepr*$^{db/+}$ 2.38 ± 0.07 mm^3; P=0.001). Relative to body weight, kidney volume was also lower in offspring of IGT *Lepr*$^{db/+}$ dams (+/+ 2.47 ± 0.07 mm^3/g, IGT *Lepr*$^{db/+}$ 2.27 ± 0.06 mm^3/g; P=0.032).

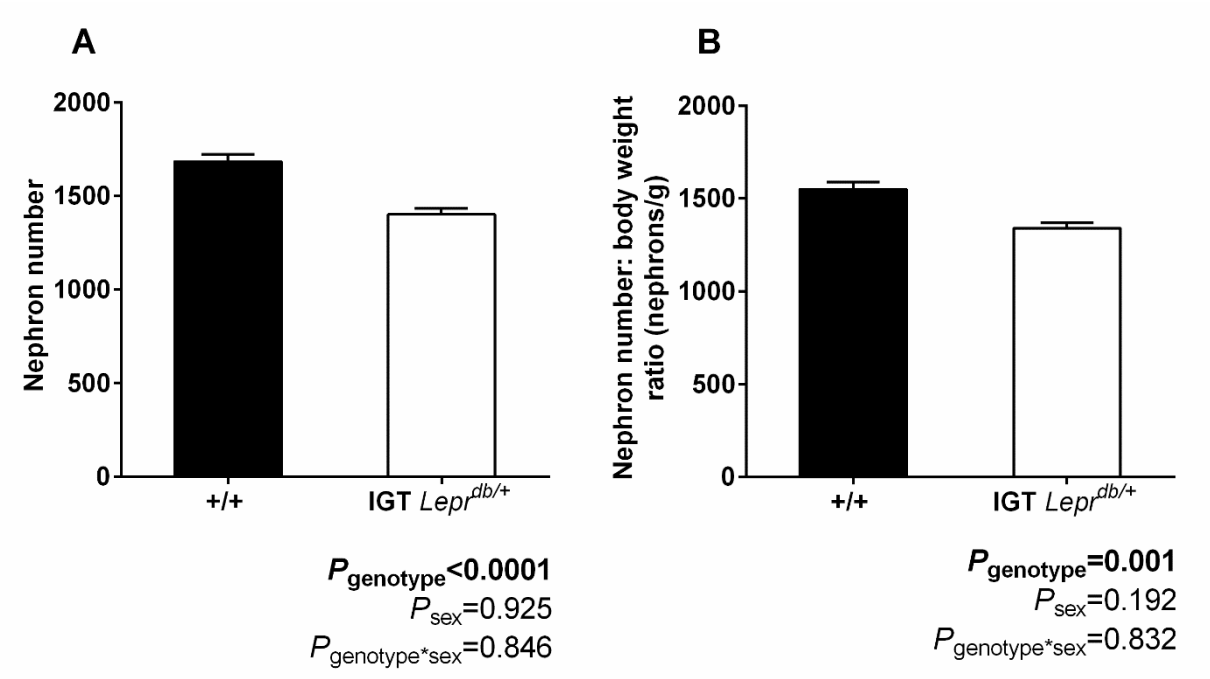


Figure 4.25 Nephron number and nephron number: body weight ratio at E18.5
A Total nephron number and **B** nephron number: body weight ratio of offspring of +/+ dams and IGT *Lepr*$^{db/+}$ dams at E18.5. Analysis by two-way ANOVA with maternal genotype and sex as variables, weighted for litter using a linear mixed model. Values are mean ± SEM. *n*(+/+)=14 kidneys from 7 litters, *n*(IGT *Lepr*$^{db/+}$)=19 kidneys from 10 litters.

4.4.6.4 Kidney development in postnatal offspring of *Lepr*$^{db/+}$ dams with impaired glucose tolerance

4.4.6.4.1 Offspring of IGT *Lepr*$^{db/+}$ dams have a low nephron endowment at PN21

At PN21, offspring of IGT *Lepr*$^{db/+}$ dams had 14% fewer nephrons than offspring of +/+ dams (+/+ 18,598 ± 378 nephrons, IGT *Lepr*$^{db/+}$ 16,042 ± 401 nephrons; *P*<0.0001; **Figure 4.26A**). This is comparable to the 17% deficit in nephron number observed at E18.5. The nephron number to body weight ratio was also significantly lower at PN21 in offspring of IGT *Lepr*$^{db/+}$ dams, with offspring having 15% fewer nephrons per gram body weight (+/+ 2050 ± 57 nephrons/g, IGT *Lepr*$^{db/+}$ 1741 ± 61 nephrons/g; *P*=0.001; **Figure 4.26B**). While no sex difference was found at E18.5, at PN21 female offspring had on average more nephrons than males (female 18,251 ± 401 nephrons, male 16,389 ± 378 nephrons; *P*=0.002; **Figure 4.26A**) and a greater nephron number to body weight ratio (female 2025 ± 60 nephrons/g, male 1765 ± 57 nephrons/g; *P*=0.004; **Figure 4.26B**). Representative images of kidneys of offspring of +/+ and IGT *Lepr*$^{db/+}$ dams are presented in **Figure 4.27**.

As outlined in **Table 4.10**, there was no difference in kidney weight or kidney volume between offspring of +/+ and *Lepr*$^{db/+}$ dams. Despite a nephron deficit there was no evidence of glomerular hypertrophy in offspring of IGT *Lepr*$^{db/+}$ dams (mean glomerular volume and total glomerular volume; **Table 4.10**).

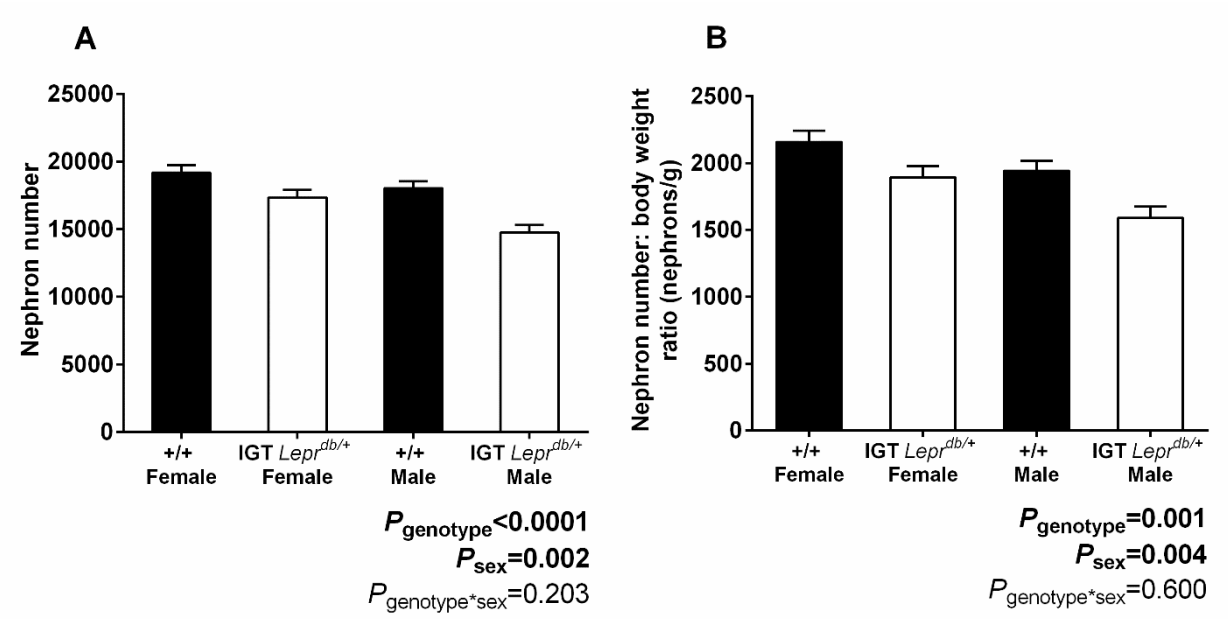


Figure 4.26 Nephron endowment and nephron number: body weight ratio at PN21

A Total nephron number and **B** nephron number: body weight ratio of offspring of +/+ and IGT *Lepr*$^{db/+}$ dams at PN21. Data analysed by two-way ANOVA with maternal genotype and sex as variables. Values are mean ± SEM. *n*(+/+ female)=7 kidneys from 7 litters, *n*(+/+ male)=9 kidneys from 9 litters, *n*(IGT *Lepr*$^{db/+}$ female)= 7 kidneys from 7 litters, *n*(IGT *Lepr*$^{db/+}$ male)= 7 kidneys from 7 litters.

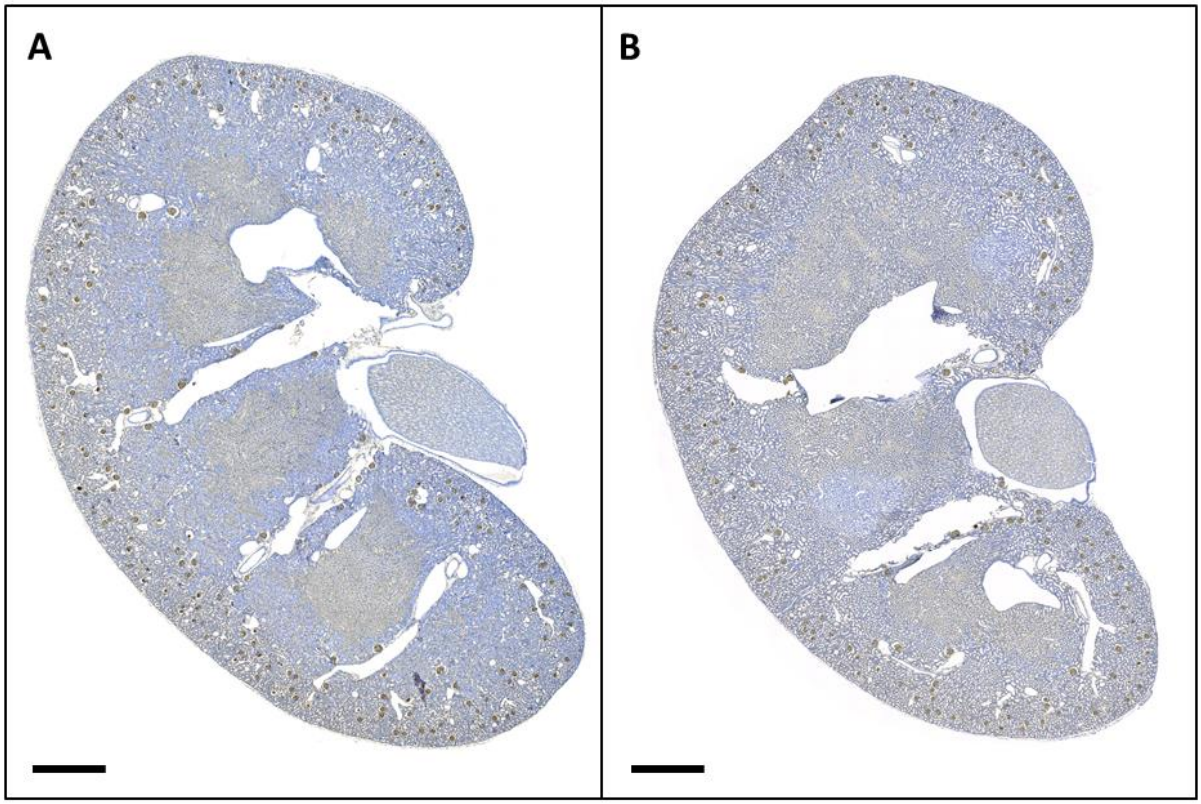


Figure 4.27 Representative images of PN21 kidney sections

Representative images of sectioned PN21 kidneys from offspring of **A** +/+ and **B** *Lepr*$^{db/+}$ dams. Sections were histochemically stained for PNA and counterstained with Haematoxylin. Scale bar = 1 mm.

Table 4.10 Offspring kidney parameters at PN21

Kidney weight, kidney volume, glomerular volume and renal corpuscle volume in offspring of +/+ and IGT *Lepr*$^{db/+}$ dams at PN21.

	+/+	IGT *Lepr*$^{db/+}$	$P_{genotype}$	P_{sex}	$P_{genotype*sex}$
Right kidney weight, mg	103.4 ± 3.7	104.8 ± 3.9	0.799	0.618	0.190
Left kidney weight, mg	96.4 ± 3.9	101.6 ± 4.1	0.363	0.981	0.989
Total kidney weight, mg	199.8 ± 7.4	206.4 ± 7.8	0.541	0.811	0.500
Kidney weight: body weight, mg/g	22.1 ± 1.0	22.4 ± 1.1	0.841	0.641	0.699
Kidney volume, mm^3	45.4 ± 2.1	41.9 ± 2.3	0.269	0.360	0.157
Mean glomerular volume, x10^{-4} mm^3	0.57 ± 0.03	0.59 ± 0.03	0.696	0.876	0.283
Mean corpuscle volume, x10^{-4} mm^3	0.93 ± 0.05	0.95 ± 0.05	0.866	0.771	0.279
Total glomerular volume, mm^3	1.06 ± 0.05	0.95 ± 0.06	0.166	0.190	0.129
Total corpuscle volume, mm^3	1.73 ± 0.08	1.51 ± 0.09	0.085	0.349	0.099

Analysis by two way ANOVA, incorporating maternal genotype and sex. Values are mean ± SEM. *n*(+/+)=16 kidneys from 9 litters, *n*(IGT *Lepr*$^{db/+}$)=14 kidneys from 7 litters.

4.4.6.5 Kidney development in offspring of *Lepr*$^{db/+}$ dams with normal glucose tolerance

To assess the effect of maternal genotype on offspring kidney development, nephron number was determined in E18.5 offspring of glucose tolerant *Lepr*$^{db/+}$ dams (NGT *Lepr*$^{db/+}$; E17.5 glucose AUC comparable to +/+ dams; AUC < 877 mmol/l.min).

4.4.6.5.1 Offspring of glucose tolerant and glucose intolerant *Lepr*$^{db/+}$ dams are of similar size

There was no difference in body weight or placental weight between offspring of +/+ dams, NGT *Lepr*$^{db/+}$ dams and IGT *Lepr*$^{db/+}$ dams (**Table 4.11**). The fetal: placental weight ratio, crown rump length and head diameter were also comparable across groups.

Table 4.11 Growth and size parameters of offspring of +/+ dams, NGT *Lepr*$^{db/+}$ dams and IGT *Lepr*$^{db/+}$ dams

	+/+	NGT *Lepr*$^{db/+}$	IGT *Lepr*$^{db/+}$	P_{group}	P_{sex}	$P_{group*sex}$
Body weight, mg	1055.1 ± 20.8	1049.0 ± 26.2	1045.4 ± 19.7	0.944	**0.002**	0.665
Placental weight, mg	91.7 ± 3.5	101.8 ± 4.2	99.3 ± 3.2	0.161	**0.002**	0.620
Fetal: placental weight ratio	11.5 ± 0.3	10.5 ± 0.4	10.6 ± 0.3	0.109	0.626	0.712
Crown rump length, mm	21.0 ± 0.2	21.2 ± 0.3	21.0 ± 0.	0.905	**<0.0001**	0.637
Head diameter, mm	11.2 ± 0.1	10.9 ± 0.1	11.2 ± 0.1	0.133	**0.031**	0.365

Analysis by two-way ANOVA with maternal genotype and sex as variables, weighted for litter using a linear mixed model. Values are mean ± SEM. *n*(+/+)=40 embryos from 7 litters, *n*(NGT *Lepr*$^{db/+}$)=18 embryos from 5 litters. *n*(IGT *Lepr*$^{db/+}$)=33 embryos from 10 litters. Only +/+ embryos were analysed.

4.4.6.5.2 Offspring of glucose tolerant *Lepr*$^{db/+}$ dams have a normal nephron number

As shown in **Figure 4.28A**, offspring of NGT *Lepr*$^{db/+}$ dams had a similar nephron number as offspring of +/+ dams (+/+ 1684 ± 37 nephrons, NGT *Lepr*$^{db/+}$ 1640 ± 50 nephrons; *P*=0.479), and significantly more nephrons than offspring of IGT *Lepr*$^{db/+}$ dams (IGT *Lepr*$^{db/+}$ 1391 ± 32 nephrons, *P*<0.0001). The nephron number: body weight ratio did not differ between +/+ and NGT *Lepr*$^{db/+}$ embryos (+/+ 1.55 ± 0.04 glomeruli/mg, NGT *Lepr*$^{db/+}$ 1.56 ± 0.06 glomeruli/mg *P*=0.841). Offspring of NGT *Lepr*$^{db/+}$ dams also had a significantly greater nephron number: body weight ratio than offspring of IGT *Lepr*$^{db/+}$ dams (NGT *Lepr*$^{db/+}$ 1561 ± 62 nephrons/g, IGT *Lepr*$^{db/+}$ 1340 ± 40 nephrons/g; *P*=0.002). Kidney volume was comparable between offspring of +/+ and NGT *Lepr*$^{db/+}$ dams (+/+ 2.70 ± 0.07 mm^3, NGT *Lepr*$^{db/+}$ 2.55 ± 0.10 mm^3; *P*=0.227) and did not differ between NGT *Lepr*$^{db/+}$ and IGT *Lepr*$^{db/+}$ embryos (NGT *Lepr*$^{db/+}$ 2.55 ± 0.10 mm^3, IGT *Lepr*$^{db/+}$ 2.38 ± 0.07 mm^3; *P*=0.156) (**Figure 4.28B**). However, kidney volume was smaller in IGT *Lepr*$^{db/+}$ embryos than in +/+ embryos (*P*=0.002).

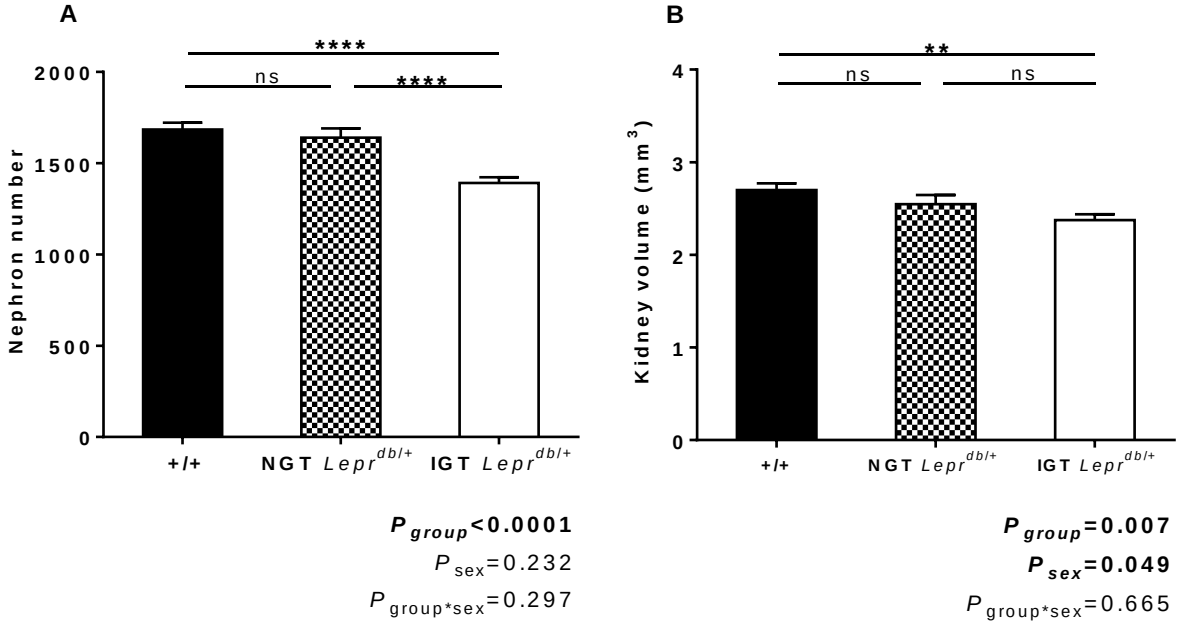


Figure 4.28 Nephron number and kidney volume in offspring of NGT *Lepr*$^{db/+}$ dams at E18.5
A Total nephron number and **B** kidney volume of offspring of +/+ dams, NGT *Lepr*$^{db/+}$ dams and IGT *Lepr*$^{db/+}$ dams at E18.5. Analysis by two-way ANOVA for maternal group and offspring sex. Post-hoc analysis by Fisher's least significant difference. Values are mean ± SEM. *n*(+/+)=14 kidneys from 7 litters, *n*(NGT *Lepr*$^{db/+}$ dams)=8 kidneys from 5 litters, *n*(IGT *Lepr*$^{db/+}$ dams)=19 kidneys from 10 litters. ***P*<0.01, **** *P*<0.0001.

4.4.6.5.3 Correlation and regression analyses between maternal and offspring parameters and kidney development at E18.5

The association between E17.5 maternal glucose tolerance (including +/+, NGT *Lepr*$^{db/+}$ and IGT *Lepr*$^{db/+}$ dams) and offspring nephron endowment at E18.5 was assessed by Spearman's rank co-efficient, presented in **Table 4.12**. Offspring nephron endowment was found to have a significant positive association with kidney volume, and a significant negative association with maternal glucose AUC, maternal weight at E0.5 and E17.5 and maternal weight gain. Interestingly, maternal weight at E17.5 was negatively associated with offspring body weight at E18.5. There were no other associations with offspring body weight.

A multiple regression analysis was performed relating offspring nephron number at E18.5 to maternal glucose AUC at E17.5, maternal weight at E17.5 and fetal body weight. These variables explained 46% of the variation in offspring nephron number (R^2=0.457, *P*=0.0001). Maternal glucose AUC had a significant negative regression weight (β=-0.642, *P*=0.0001) indicating that offspring of dams with glucose intolerance in late gestation were expected to have fewer nephrons at E18.5. Maternal weight at E17.5 and fetal weight at E18.5 did not contribute significantly to the multiple regression model.

Table 4.12 Spearman's rank coefficients for associations between nephron number at E18.5 and independent variables in +/+, NGT *Lepr*$^{db/+}$ and IGT *Lepr*$^{db/+}$ dams

		Offspring body weight	Offspring nephron endowment	Offspring kidney volume	Maternal weight gain	Maternal weight E0.5	Maternal weight E17.5
Offspring nephron endowment	**rho**	0.186					
	P	0.243					
Offspring kidney volume	**rho**	0.158	**0.530**				
	P	0.322	**0.0004**				
Maternal weight gain	**rho**	-0.285	**-0.575**	-0.247			
	P	0.090	**0.0002**	0.147			
Maternal weight E0.5	**rho**	-0.308	**-0.512**	-0.315	**0.770**		
	P	0.060	**0.001**	0.054	**<0.0001**		
Maternal weight E17.5	**rho**	**-0.348**	**-0.508**	**-0.334**	**0.815**	**0.967**	
	P	**0.030**	**0.001**	**0.038**	**<0.0001**	**<0.0001**	
Maternal glucose AUC E17.5	**rho**	-0.238	**-0.741**	**-0.341**	**0.689**	**0.731**	**0.558**
	P	0.134	**<0.0001**	**0.029**	**<0.0001**	**0.0006**	**<0.0001**

Spearman's rho (top value), *P* value (bottom value). Negative values indicate a negative association. Values in bold are statistically significant.

The negative association between maternal glucose and offspring nephron number, including offspring of *Lepr*$^{db/+}$ dams with normal glucose tolerance, is highlighted in **Figure 4.29A** and **Figure 4.29B**. These plots show that as maternal glucose intolerance increases offspring nephron number is reduced. The association between maternal weight at E0.5 and offspring nephron number at E18.5 was analysed. **Figure 4.29C** shows that as maternal weight increases offspring nephron number is reduced.

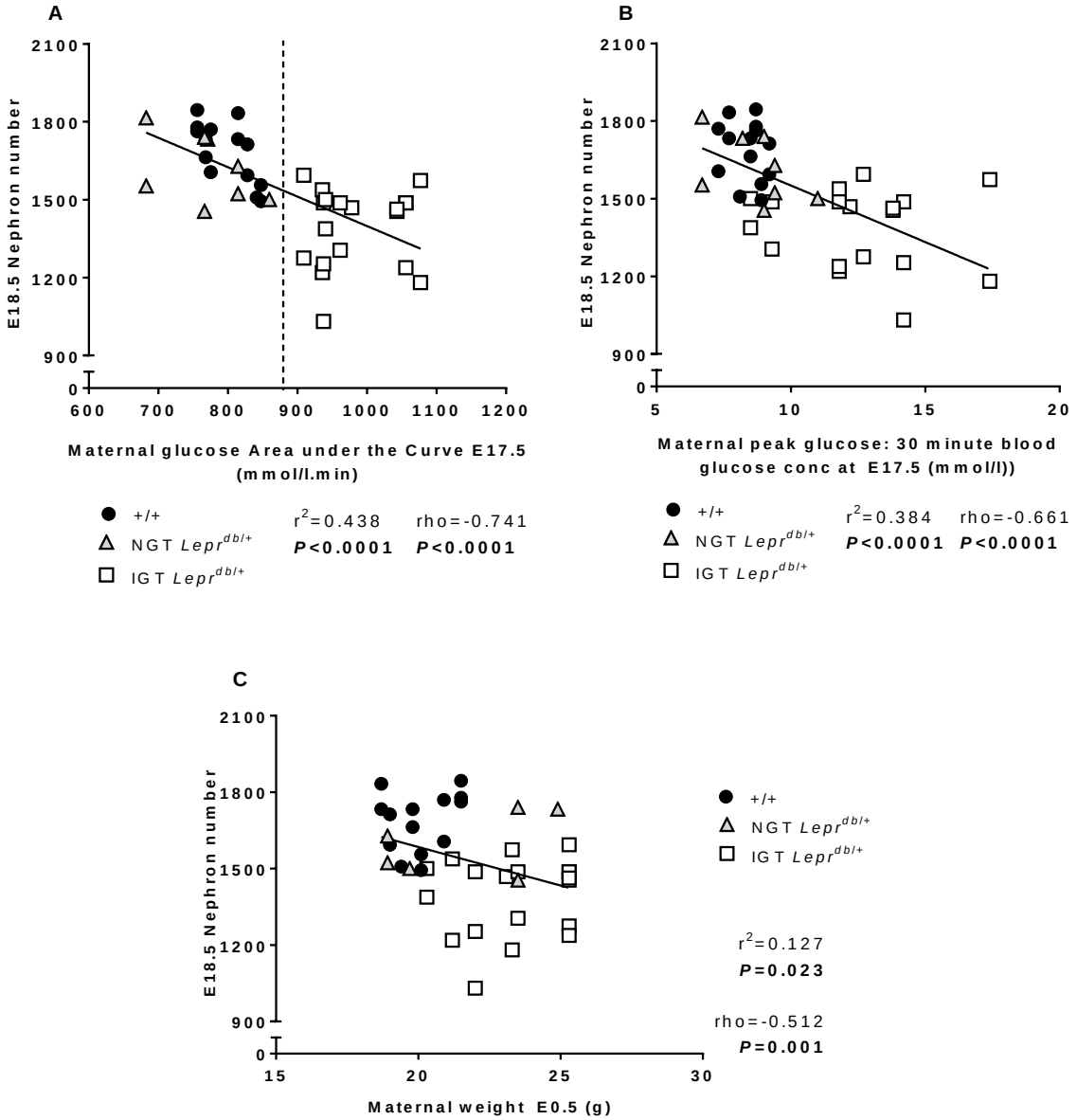


Figure 4.29 Associations between offspring nephron number at E18.5 and maternal glucose tolerance at E17.5, peak maternal glucose at E17.5 and maternal weight at E0.5, including offspring of NGT *Lepr*$^{db/+}$ dams

Nephron number in E18.5 offspring of +/+, NGT *Lepr*$^{db/+}$ and IGT *Lepr*$^{db/+}$ dams plotted against **A** maternal glucose AUC at E17.5, **B** peak maternal glucose levels (30 minutes after a glucose load) at E17.5 and **C** maternal body weight at E0.5. Dashed line indicates cut-off criteria used to identify glucose tolerant and glucose intolerant *Lepr*$^{db/+}$ dams. Solid line indicates line of best fit for combined data. Data analysed by Spearman's rho and linear regression. *n*(NFD NGT)=16 kidneys from 8 litters, *n*(HFD NGT)=12 kidneys from 7 litters, *n*(HFD IGT)=12 kidneys from 6 litters.

4.5 Discussion

In the present chapter, leptin receptor deficient *Lepr*$^{db/+}$ mice and their offspring were studied to investigate the effect of gestational diabetes on kidney development. In contrast to what has been reported in the literature, *Lepr*$^{db/+}$ dams exhibited pre-gestational glucose intolerance. Accordingly, in the present study *Lepr*$^{db/+}$ mice served as a model of pre-gestational Type 2 diabetes. GTTs performed in late gestation revealed incomplete penetrance of glucose intolerance in *Lepr*$^{db/+}$ dams; an additional phenotype not previously described. Offspring of glucose intolerant *Lepr*$^{db/+}$ dams were of normal body weight, but had a deficit in nephron endowment that was established early in nephrogenesis. These results and their implications are discussed below.

4.5.1 The *misty* mutation

The *misty* mutation was originally incorporated into stocks of *Lepr*^{db} mice to aid in the identification of *Lepr*$^{db/db}$, *Lepr*$^{db/+}$ and *Lepr*$^{+/+}$ mice prior to modern genotyping techniques (Coleman, 1978). Although a direct assay is now available to genotype at *Lepr* (Horvat and Bünger, 1999), The Jackson Laboratory continues to stock populations of C57BLKS/J *Lepr*^{db} mice with *misty* maintained in repulsion. Publications that describe gestational diabetes in *Lepr*$^{db/+}$ mice have used *misty* mice as controls (Kaufmann et al., 1981, Ishizuka et al., 1999, Yamashita et al., 2001, Shao et al., 2002, Yamashita et al., 2003, Stanley et al., 2011). Together, these studies report hyperphagia, increased gestational weight gain and glucose intolerance in late gestation in *Lepr*$^{db/+}$ dams compared with wild type *misty* dams.

Unexpectedly, in the present study the colony of *Lepr*$^{db/+}$ *misty* mice exhibited normal glucose tolerance. When glucose profiles were assessed on an individual basis, one third of *Lepr*$^{db/+}$ *misty* mice displayed an elevated peak glucose level, indicating incomplete penetrance of glucose intolerance. It is unclear why incomplete penetrance of glucose intolerance was observed, but it is hypothesised that it is related to maternal weight gain and food intake, which were similar between +/+ *misty* dams and *Lepr*$^{db/+}$ *misty* dams. Given that an inability to suppress feeding behaviour is a key feature of obese and diabetic *Lepr*$^{db/db}$ mice, it follows that *Lepr*$^{db/+}$ dams without hyperphagia or increased gestational weight gain are likely to display normal glucose tolerance.

When *misty* was originally introduced into the *Lepr*^{db} colony, it was assumed that *misty* had no independent effects on traits affected by *Lepr*^{db}. However, Truett et al. (1998) reported reduced body length, body weight and adiposity in *misty* mice. The use of mice segregating *misty* in studies of growth and metabolism, such as in gestational diabetes, is evidently undesirable and confounding given the imbalance in the number of *misty* alleles in *Lepr*$^{db/+}$ and wild type mice. Subsequently, further analysis of +/+ *misty* and *Lepr*$^{db/+}$ *misty* dams was abandoned and *misty* was bred out of the *Lepr*$^{db/+}$ colony. It is interesting, and at the same time disappointing, that only one study examining *Lepr*$^{db/+}$ mice in

pregnancy has avoided *misty* following the publication by Truett et al. (1998) (Harrod et al., 2011). This failure of many studies to account for the confounding effects of *misty* has without doubt led to the publication of many misleading reports.

4.5.2 Pre-gestational glucose intolerance

All previous publications of *Lepr*$^{db/+}$ mice report normal basal glucose levels and normal glucose handling prior to pregnancy (Kaufmann et al., 1981, Ishizuka et al., 1999, Yamashita et al., 2001, Yamashita et al., 2003, Stanley et al., 2011). In the present chapter *Lepr*$^{db/+}$ mice without *misty* were found to exhibit normal fasting glucose but, in contrast, exhibited elevated glucose AUC values and mild postprandial hyperglycaemia prior to pregnancy (Yamashita et al., 2003, Stanley et al., 2011). The greater body weight observed in *Lepr*$^{db/+}$ mice at E0.5 does differ to previous reports (Kaufmann et al., 1981, Yamashita et al., 2001, Yamashita et al., 2003, Lambin et al., 2007, Stanley et al., 2011) and it may be this novel phenotype that explains the pre-gestational glucose intolerance. The finding that IGT *Lepr*$^{db/+}$ mice were heavier than NGT *Lepr*$^{db/+}$ mice at E0.5 further supports this hypothesis. The increase in body weight at E0.5 was seen in *Lepr*$^{db/+}$ mice both with and without *misty* and cannot be attributed to the loss of *misty* from the colony. Unfortunately, pre-gestational GTTs were not performed in *misty* mice and it is possible that pre-gestational glucose intolerance in *Lepr*$^{db/+}$ mice is related to the removal of *misty*.

4.5.3 Incomplete penetrance of glucose intolerance in late gestation

Removing *misty* from the colony unfortunately did not improve the penetrance of glucose intolerance in late gestation. Previously, Harrod et al. (2011) recognised the confounding effects of *misty* and used C57BLKS/J mice as a control group. They also observed no overall difference in glucose tolerance between *Lepr*$^{db/+}$ and C57BLKS/J mice, although they made no mention of individual glucose profiles in *Lepr*$^{db/+}$ dams. It is unclear why incomplete penetrance of glucose intolerance in *Lepr*$^{db/+}$ dams was observed in this study. Previously, Ishizuka et al. (1999) cited “virtually 100% penetrance in db/+ mice (30 minute blood glucose > 13.9mmol/l)”. Other studies make no mention of penetrance. Similar to Ishizuka et al. (1999), mice in the present study were initially selected as glucose intolerant based on an elevated 30 minute glucose concentration (systematically defined as > 9.9mmol/l). This identified 50% of *Lepr*$^{db/+}$ dams as glucose intolerant, similar to the 45% penetrance observed using glucose AUC criteria. As glucose AUC encompasses a 2 hour glucose profile rather than a single point value, this was considered to be a better representation of overall glucose handling. This does however raise the question of what is more detrimental to the fetus: marked elevations in postprandial glucose that shortly return to baseline or moderate yet prolonged postprandial hyperglycaemia.

Based on the glucose AUC value at E17.5, *Lepr*$^{db/+}$ mice were defined as glucose tolerant or glucose intolerant. Selected IGT *Lepr*$^{db/+}$ dams were found to have mild fasting hyperglycaemia and a marked elevation in 30 minute peak glucose levels as previously reported (Kaufmann et al., 1981, Ishizuka et al., 1999, Yamashita et al., 2001, Yamashita et al., 2003, Stanley et al., 2011). Surprisingly, lower glucose curves and glucose AUC values were observed in +/+ dams in late pregnancy compared to pre-pregnancy, an observation not previously described. Mean fasting glucose levels and glucose profiles at 60, 90 and 120 minutes were also lower in IGT *Lepr*$^{db/+}$ mice in pregnancy compared to before pregnancy. However, this was coupled with a dramatic increase in 30 minute peak glucose levels which contributed to stable glucose AUC values before and during gestation. These factors contribute to the marked glucose intolerance observed in *Lepr*$^{db/+}$ dams compared to +/+ dams in late gestation.

Previously, Harrod et al. (2011) used high fat feeding to induce glucose intolerance in *Lepr*$^{db/+}$ mice. *Lepr*$^{db/+}$ mice were fed a high fat diet (45% digestible energy from fat) and were compared to wild type mice fed a normal fat diet (6% digestible energy from fat). While the length of time on diet was not specified, *Lepr*$^{db/+}$ mice displayed normal glucose profiles before mating but profound glucose intolerance in pregnancy. High fat feeding in the present study may have improved the penetrance of glucose intolerance in *Lepr*$^{db/+}$ dams. However, as described in Chapter 2 high fat feeding would have added a level of complexity when deducing the isolated effects of dietary components as well as maternal high glucose and a mutation in the leptin receptor on offspring outcomes. For this reason a high fat dietary protocol was avoided.

Glucose intolerant *Lepr*$^{db/+}$ dams were found to share other features reported in previous publications of gestational diabetes: IGT *Lepr*$^{db/+}$ dams had excess maternal weight gain, hyperphagia and hyperinsulinaemia compared with +/+ dams (Ishizuka et al., 1999, Yamashita et al., 2001, Yamashita et al., 2003, Stanley et al., 2011). It is important to note that food intake was elevated in *Lepr*$^{db/+}$ mice regardless of glucose tolerance status. Pregnancy weight gain was also elevated but was confounded by an increase in litter size. However, following adjustment for uterus weight (a measure of total conceptus and placental weight), weight gain was significantly elevated in IGT *Lepr*$^{db/+}$ dams. This likely indicates a greater increase in fat mass during gestation as previously reported by Ishizuka et al. (1999) and Yamashita et al. (2001).

The ability to predict which *Lepr*$^{db/+}$ mouse would exhibit impaired glucose tolerance in pregnancy was difficult. Compared with NGT *Lepr*$^{db/+}$ dams, IGT *Lepr*$^{db/+}$ dams did not differ in weight gain, age or food intake, and were only slightly 5% heavier at E0.5 and in late gestation. Pre-pregnancy glucose profiles were also similar in NGT and IGT *Lepr*$^{db/+}$ dams. The low penetrance of glucose intolerance in late

gestation, coupled with the inability to predict which dam would display glucose intolerance, discourages future use of this strain to study maternal glucose intolerance.

4.5.4 Postnatal glucose tolerance

Glucose intolerance was not maintained in the postnatal period. As dams exhibited glucose intolerance prior to pregnancy, it was surprising that this phenotype was not present after birth at PN4 or PN21. In order to test glucose levels in the postnatal period, dams were fasted for 6 hours and then subjected to a 2 hour GTT. Pups were kept with the dam during the test as it was considered unethical to remove the litter for such an extended period of time. It is therefore plausible that lactation interfered with the results obtained. Weaning-age pups were kept with the dam during the PN21 GTT. In hindsight, a more accurate measure of glucose tolerance would have been obtained if pups were removed from the dam at this time. Previously, only one study investigating gestational diabetes in *Lepr*$^{db/+}$ dams has measured postnatal glucose tolerance. In agreement with the present study, Kaufmann et al. (1981) found normal glucose tolerance in *Lepr*$^{db/+}$ *misty* dams 1 month after delivery. However, 3 hour glucose levels were significantly elevated. Maternal postpartum weight was not reported. In the present study *Lepr*$^{db/+}$ dams were heavier than +/+ dams at PN4 and PN21 which was also the case for their pre-pregnancy weight.

4.5.5 Offspring body weight

In contrast to what is reported in published *Lepr*$^{db/+}$ studies and despite fetal hyperinsulinaemia, offspring macrosomia was not observed at E18.5. Previous studies report offspring macrosomia at E18.5 and birth (Kaufmann et al., 1981, Ishizuka et al., 1999, Yamashita et al., 2001, Yamashita et al., 2003, Stanley et al., 2011). In the present study offspring were heavier at PN2, but this included both +/+ pups and *Lepr*$^{db/+}$ pups of *Lepr*$^{db/+}$ dams. The increase in pup weight at PN2 is not likely to represent an increase in body weight of +/+ pups of *Lepr*$^{db/+}$ dams in response to maternal hyperglycaemia, as there was no difference in fetal weight at E18.5. While *Lepr*$^{db/+}$ pups of *Lepr*$^{db/+}$ dams were also not heavier than +/+ pups at E18.5, body size may have increased in the early postnatal period independent of the maternal environment.

Two *Lepr*$^{db/+}$ studies have reported normal pup weight. Bobadilla et al. (2010) found no significant difference in body weight at E18.5. This was reported in *Lepr*$^{db/+}$ dams with normal non-fasting glucose levels however fasting glucose and glucose tolerance were not measured. Lawrence et al. (1989) used both *misty* mice and C57Bl6/KSJ mice as controls. They found macrosomia in pups of *Lepr*$^{db/+}$ dams, but only when compared to pups of *misty* dams and not to pups of C57Bl6/KSJ dams. Maternal blood glucose levels were not reported.

The increase in peak glucose levels between +/+ and *Lepr*$^{db/+}$ dams in late gestation in this study is comparable to that reported in other studies which observed neonatal macrosomia (Kaufmann et al., 1981, Ishizuka et al., 1999, Yamashita et al., 2001, Yamashita et al., 2003, Stanley et al., 2011). The absence of neonatal macrosomia in the present chapter may instead be related to: (i) the uncharacteristically greater litter size in *Lepr*$^{db/+}$ pregnancies, a finding that has not been described before and which likely impacts on nutrient availability and fetal growth; (ii) past studies which fail to discern if pups with *Lepr*$^{db/+}$ genotype are included in analysis (Kaufmann et al., 1981, Stanley et al., 2011); (iii) the general difficulty in obtaining macrosomia in mice due to their immaturity at birth and paucity of fat tissue (Widdowson, 1950); or (iv) a loss of phenotype following the removal of *misty* from the population.

4.5.6 Maternal and fetal insulin and leptin

Maternal hyperinsulinaemia and hyperleptinaemia are common features of *Lepr*$^{db/+}$ dams. Circulating leptin levels are reported to increase dramatically in pregnancy in both +/+ and *Lepr*$^{db/+}$ mice (30-40 fold), yet are even greater in *Lepr*$^{db/+}$ dams in late gestation (Ishizuka et al., 1999, Yamashita et al., 2001). While basal leptin levels were not measured in the present study, a 2-fold elevation in fasting leptin levels was observed in late gestation and can be attributed to leptin receptor deficiency. Hyperleptinaemia was associated with an almost 4-fold increase in fasting insulin levels. Hyperinsulinaemia, reduced insulin responses and insulin resistance are reported in other studies of pregnant *Lepr*$^{db/+}$ mice (Ishizuka et al., 1999, Yamashita et al., 2001, Shao et al., 2002, Yamashita et al., 2003). Insulin resistance is the underlying pathophysiological feature of human gestational diabetes and supports the use of *Lepr*$^{db/+}$ mice to model gestational diabetes.

Fetal leptin and insulin levels have been poorly reported in previous studies of *Lepr*$^{db/+}$ mice. Yamashita et al. (2001) found increased fetal serum leptin and placental leptin in offspring exposed to maternal glucose intolerance. Lawrence et al. (1989) reported increased serum insulin and decreased pancreatic insulin content in offspring of *Lepr*$^{db/+}$ dams assumed to have gestational diabetes. Both of these studies combined serum from +/+ and *Lepr*$^{db/+}$ offspring of *Lepr*$^{db/+}$ dams. In the present study, +/+ fetal offspring of *Lepr*$^{db/+}$ dams were hyperinsulinaemic. This is suggestive of fetal pancreatic involvement in response to hyperglycaemia. +/+ fetuses of *Lepr*$^{db/+}$ dams were also hyperleptinemic. Given the small amount of adipose tissue present in fetal mice, the increase in fetal leptin is likely to be reflective of the maternal environment or increased placental leptin production. However, it is uncertain whether maternal leptin crosses the placenta and whether the murine placenta expresses leptin (Hoggard et al., 2001, Malik et al., 2005, Forhead and Fowden, 2009).

4.5.7 Kidney development

Previous studies investigating nephron number in rodent models of maternal diabetes focus on the Type 1 diabetes model of STZ-induced selective toxicity of pancreatic islet β cells, resulting in severe fasting hyperglycaemia. These studies reported a 15-40% deficit in nephron endowment that is proportionate to the magnitude of maternal fasting glucose (fasting values ranging from 20-28 mmol/l in STZ-treated dams vs. 5-11mmol/l in control dams), with reductions in offspring birth weight exacerbating (yet confounding) a nephron deficit (Amri et al., 1999, Nehiri et al., 2008, Tran et al., 2008, Hokke et al., 2013). These studies clearly show that chronic exposure to severe fasting maternal hyperglycaemia is detrimental to nephron progenitor populations and ureteric epithelium.

In the present study, offspring of glucose intolerant *Lepr*$^{db/+}$ dams had a 15% deficit in nephron number. This deficit was observed early in nephrogenesis at E18.5, when only a small proportion of PNA-positive glomeruli (<10%) had formed. The reduction in glomerular number was also observed at PN21 following the completion of nephron formation, demonstrating a permanent nephron deficit in offspring. The comparable nephron deficit observed in this study and STZ studies indicates that intermittent episodes of hyperglycaemia may be as damaging to the developing kidney as chronic fasting hyperglycaemia.

This is supported by Amri et al. (1999) who gave continuous glucose infusion to pregnant rats from E12 to E16, identifying that a short period of hyperglycaemia during the early stages of kidney development could induce a permanent deficit in nephron endowment. In their study, one group of glucose-infused dams were found to have a constant 2-fold increase in glucose levels over the 4 day protocol (11mmol/l vs. 5mmol/l in controls) that was associated with normal offspring birth weight and normal nephron endowment. A second group of dams had similar hyperglycaemia but with a transient elevation in glucose levels at E13 (~17mmol/l) that was associated with normal offspring birth weight but a 20% deficit in nephron endowment.

Together, studies examining kidney development in a high glucose intrauterine environment indicate that any marked increase in maternal glucose (whether it be chronically elevated fasting glucose seen in STZ studies, transient hyperglycaemia seen in the glucose infusion study, or postprandial hyperglycaemia seen in the present study) can permanently alter the number of nephrons formed. It is surprising that offspring of glucose-infused dams with a 2-fold increase in glucose levels did not have perturbed nephron endowment (Amri et al. 1999). This finding may indicate a threshold in maternal glucose required to elicit damage in the developing kidney. Further investigation of kidney development in offspring exposed to mild hyperglycaemia is required.

Nephron number increases rapidly in mice in the postnatal period, with nephrogenesis ceasing at PN5. In the present study, similar deficits in nephron number were observed before birth, at a time associated with profound maternal glucose intolerance, and in the postnatal period, when glucose tolerance was similar between groups. This indicates that exposure to maternal glucose intolerance during early renal development perturbs and induces damage in the kidney that cannot be recovered after birth, despite an exponential increase in nephron formation. As nephron formation occurs adjacent to ureteric tips, and, given the iterative nature of branching morphogenesis, a small reduction in ureteric branching can have a substantial influence on the capacity for nephron formation. This is demonstrated in the glucose infusion study of Amri et al. (1999). In their study, 1 day of transiently elevated hyperglycaemia at E13 was sufficient to induce a 20% deficit in nephron endowment. At this gestational age nephrogenesis has just commenced however ureteric epithelium is undergoing extensive branching. Studies by Cebrián et al. (2004) and Short et al. (2014) report that 6-7 branch generations (over half) have occurred by E14, meaning the deficit in nephron number reported by Amri et al. (1999) is most likely due to reduced branching events. The length of exposure to an intrauterine insult, and when in gestation the embryo is exposed to a high glucose environment, can therefore have important implications for the developing kidneys.

In the present study, GTTs were performed twice during gestation. This revealed mild glucose intolerance and fasting hyperglycaemia at E12.5 and profound glucose intolerance and mild fasting hyperglycaemia at E17.5 in IGT *Lepr*$^{db/+}$ dams. GTTs were not performed between these two time points and therefore the length of time embryonic offspring were exposed to these marked postprandial elevations in glucose is unknown. Ureteric tree development was not measured in this study. However, based on previous studies that report a combined deficit in ureteric branching morphogenesis and nephron endowment (Amri et al., 1999, Hokke et al., 2013), ureteric tree development is likely to be reduced in offspring of glucose intolerant *Lepr*$^{db/+}$ dams. The ureteric epithelium may also be indirectly affected. A recent study by Cebrian et al. (2014) found that the genetic ablation of nephron progenitor cells resulted in a subsequent reduction in ureteric branching and a permanent deficit in nephron endowment. It is therefore plausible that a high glucose milieu may affect ureteric epithelia as well as nephron progenitor cell populations.

One advantage of *Lepr*$^{db/+}$ dams exhibiting incomplete penetrance of glucose intolerance in late gestation was the ability to assess nephron number in offspring of *Lepr*$^{db/+}$ dams with normal glucose tolerance. This enabled the effects of maternal genotype to be isolated from maternal glucose. Offspring of NGT *Lepr*$^{db/+}$ dams had normal nephron number. The deficit in nephron number observed in offspring of IGT *Lepr*$^{db/+}$ dams is therefore attributed to maternal glucose intolerance in late gestation.

4.5.8 Concluding remarks

In conclusion, the *Lepr*$^{db/+}$ phenotype reported in this study highlights the difficulty in obtaining a reliable and consistent mouse model of human gestational diabetes. The present study does, however, present a valuable model of pre-gestational diabetes, as the IGT *Lepr*$^{db/+}$ mouse shares features of the increasingly common human condition; maternal overweight, insulin resistance and glucose intolerance before and during pregnancy. Moderate postprandial increases in glucose can have an adverse and permanent consequence on nephron endowment and these deficits are comparable to what is reported in offspring exposed to constant and severe fasting hyperglycaemia. Further studies investigating how maternal glucose intolerance induces a deficit in nephron endowment, as well as assessment of renal function and morphology in adult offspring of IGT *Lepr*$^{db/+}$ dams are warranted.

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CHAPTER FIVE

HUMAN FETAL KIDNEY VOLUME IN GESTATIONAL DIABETES

5.1 Introduction

Metanephric development in humans begins at 5 weeks gestation with nephrogenesis complete by approximately 34-36 weeks gestation (Hinchliffe et al., 1991). In a study of 400 human kidneys at autopsy, Bertram et al. (2011) reported a 13-fold range in nephron number. This wide variation in nephron number in subjects without kidney disease may relate to the degree of nephron loss in postnatal life. However, findings from several studies have indicated that much of this variation in nephron number is present at birth (Manalich et al., 2000, Zhang et al., 2008). As there is no evidence for postnatal nephrogenesis in humans (Luyckx et al., 2011), nephron endowment is ultimately established *in utero* and is therefore developmentally determined.

As described in **Section 1.8**, nephron endowment is critically dependent on a favourable intrauterine environment (Hoy et al., 2010). In response to fetal malnutrition, hypoxia or other adverse intrauterine stimuli, development of the brain and heart are 'spared' by fetal circulatory redistribution, at the expense of other organs including the developing kidneys (Lumbers et al., 2001). Human studies have shown a strong correlation between birth weight and nephron number (Hughson et al., 2003, Bertram et al., 2011), with each kilogram increase in birth weight associated with an additional 200,000 to 300,000 nephrons per kidney. Low nephron number has been reported in human studies of low birth weight and intrauterine growth restriction (Hinchliffe et al., 1992, Manalich et al., 2000). Maternal factors that are associated with low nephron number and smaller kidneys in humans and animal models include protein deficiency, vitamin A and other micronutrient deficiencies, hypoxia, infections, alcohol consumption, certain antibiotics, steroids and hyperglycaemia (reviewed by Lelièvre-Pégorier and Merlet-Bénichou (2000), Hoy et al. (2010), Luyckx and Brenner (2010)). Interestingly, not all of these factors are associated with low birth weight.

The implication of a congenital nephron deficit was first hypothesised by Brenner et al. (1988) who suggested a link between low nephron number and hypertension and renal disease in adulthood. A low nephron number was postulated to be associated with low filtration surface area, sodium retention, systemic hypertension, glomerular hyperfiltration and injury and ultimately renal disease (see **Section 1.8**). Since its enunciation, numerous experimental and epidemiological studies have emerged to support the Brenner hypothesis (Keller et al., 2003, Hughson et al., 2006, Luyckx et al., 2013).

Estimation of human nephron endowment is currently only feasible from autopsy tissue and for this reason very few studies have quantified nephron endowment. Previously, a direct relationship between kidney mass and total nephron number has been reported in infants and adults (Nyengaard and Bendtsen, 1992, Zhang et al., 2008). As renal mass is proportional to renal volume, kidney volume

can be used as a surrogate measure of nephron number and can be readily assessed *in vivo* using ultrasound (Nyengaard and Bendtsen, 1992, Luyckx and Brenner, 2010).

The use of ultrasound to assess fetal anatomy is part of routine antenatal care. Ultrasound enables the detection and diagnosis of abnormalities of the kidney and urinary tract, which comprise up to one third of all congenital abnormalities. Obstetric ultrasound has also been applied to monitor fetal renal growth across gestation in normal pregnancy (Gloor et al., 1997, Konje et al., 1997, Yu et al., 2000, Verburg et al., 2007). These studies report a positive association between kidney volume and gestational age, estimated fetal weight (EFW) and abdominal circumference, and show that renal growth is proportional to fetal growth. Serial ultrasound measurements have also been used to assess renal growth velocities over time. Konje et al. (1996) found an accelerative phase of maximal renal growth between 26-34 weeks gestation followed by a decelerative phase between 34-38 weeks gestation. These findings support those of Hinchliffe et al. (1991), who showed that 60% of the nephrons form in the third trimester with nephrogenesis complete at 34-36 weeks gestation. The third trimester therefore represents a critical period of kidney development. An adverse intrauterine environment during this time may adversely affect renal growth and nephron formation.

Differential renal growth patterns have been observed in SGA fetuses. Reduced renal growth velocities, smaller kidney sizes and reduced renal perfusion have been documented in hypoxic, SGA fetuses compared to appropriately grown fetuses (Vyas et al., 1989, Konje et al., 1996). Low birth weight Aboriginal children also exhibit reduced kidney volumes compared to normal birth weight Aboriginal children (Spencer et al., 2001). Interestingly, the reduced kidney volumes seen by Spencer et al. (2001) and Konje et al. (1996) were largely driven by reduced kidney depths/widths and not alterations in kidney lengths. Fetal kidney sizes have also been found to track into early childhood, with smaller third trimester kidney volumes persisting at 2 years of age. These smaller kidney volumes are accompanied by preferential fetal blood flow to the brain (Geelhoed et al., 2009).

Studies that report a reduction in renal volume are typically seen in the context of SGA or low birth weight babies. In contrast, gestational diabetes is frequently associated with fetal overgrowth. Gestational diabetes typically manifests in mid-pregnancy, although many women may not be screened until 28 weeks gestation and may not commence treatment until 30 weeks gestation. It is therefore postulated that a window of hyperglycaemia, which coincides with a period of rapid renal growth and nephron formation, may have adverse consequences for developing kidneys.

Few studies have utilised ultrasound to measure renal size in offspring of maternal diabetes. Verburg et al. (2007) found no association between maternal diabetes (pre-existing or gestational) and fetal renal volume at 30 weeks gestation. Neves et al. (2013) longitudinally measured kidney volume in a

large cohort of women with (*n*=92) and without (*n*=329) gestational diabetes, with serial ultrasounds performed between 22-38 weeks gestation. They found markedly increased fetal kidney volumes in gestational diabetic pregnancies across gestation. Interestingly, excessive fetal kidney growth was observed prior to the diagnosis of gestational diabetes and was seen despite 80% of women achieving glycaemic control. Unfortunately, fetal weights and fetal growth were not documented. In addition, a small study by Cappuccini et al. (2013) used 3D ultrasound to quantify renal cortical volume in 3 year old children. Compared to children of non-diabetic pregnancy (*n*=21), normal birth weight children of women with Type 2 diabetes and gestational diabetes (*n*=40) had reduced renal cortical volumes and albuminuria, suggesting a reduction in nephron endowment and subsequent glomerular hyperfiltration.

Maternal hyperglycaemia is recognised to stimulate fetal growth via fetal pancreatic involvement and insulin production. Whether these factors also stimulate kidney growth and nephron formation or whether they are detrimental remains unclear. Given the equivocal findings of Verburg et al. (2007), Cappuccini et al. (2013) and Neves et al. (2013) further assessment of renal growth in human maternal diabetes is warranted. Understanding how hyperglycaemia and glucose intolerance in pregnancy can affect the developing fetal kidneys is of importance considering the increasingly high prevalence of this condition.

To assess kidney size in offspring of maternal diabetes, a prospective cohort study was established at Monash Medical Centre, Monash Health, Victoria, Australia. The governing ethics committees approved the recruitment of 85 women to each of five groups: (i) women with Type 1 diabetes; (ii) women with Type 2 diabetes; (iii) women with early onset gestational diabetes (diagnosed at first antenatal visit); (iv) women with gestational diabetes (diagnosed during routine screening between 24-28 weeks); and (v) women without diabetes. This Chapter presents a comprehensive analysis of maternal and fetal outcomes for women with gestational diabetes (*n*=36) and women with normal glucose tolerance in pregnancy (*n*=42). Numbers of women recruited to the other groups (eight women with Type 1 diabetes, five women with Type 2 diabetes and seven women with gestational diabetes diagnosed early in pregnancy) are too low to allow a meaningful and informative quantitative analysis at this time.

5.2 Aim

- To assess, using data from human obstetric ultrasonography, whether gestational diabetes in pregnancy affects fetal kidney size and volume as a measure of renal sufficiency.

5.3 Hypothesis

- Compared to offspring of non-diabetic pregnancy, fetuses of women with gestational diabetes will have smaller kidney volumes (absolute or relative to body weight).

5.4 Methods

A prospective cohort study was undertaken at Monash Medical Centre, Monash Health, a large tertiary level maternity service in Melbourne, Australia. Ethics approval was granted by the Monash University Human Research Ethics Committee and the Monash Health Human Research Ethics Committee (13041B). Informed written consent was obtained from all participants.

The principle investigator of this study is Professor Euan Wallace (Carl Wood Professor of Obstetrics and Gynaecology, Monash University; Co-Head, The Ritchie Centre, Hudson Institute of Medical Research; Head of Obstetrics and Gynaecology, Monash University; Director of Obstetric Services, Monash Health). The study is coordinated by Dr. Luise Cullen-McEwen (Senior Research Fellow, Department of Anatomy and Developmental Biology, Monash University). Other associate investigators include Professor John Bertram (Head of Department and Head of Kidney Development, Programming and Disease Research Group, Department of Anatomy and Developmental Biology, Monash University), Associate Professor Carolyn Allan (Consultant Endocrinologist; Head of Endocrine Pregnancy Services, Monash Health; Senior Medical Staff, Departments of Endocrinology and Diabetes, Monash Medical Centre, Monash Health; Adjunct Clinical Associate Professor, Department of Obstetrics and Gynaecology, Monash University), Dr. Andrew Edwards (Head of Perinatal Services-Fetal Diagnostic Unit, Monash Medical Centre, Monash Health), Dr. James Armitage (Research Fellow, Department of Anatomy and Developmental Biology, Monash University), Mr Peter Coombs (Chief Sonographer, Ultrasound, Monash Health), Mrs Bethany Carr (Research Midwife, Monash Health) and myself.

Women who participated in the study underwent an obstetric ultrasound between 32-34 weeks gestation to measure fetal biometry and fetal kidney size. Maternal data and birth data were also collected.

5.4.1 Participants

Women attending the Monash Medical Centre Obstetric and Diabetes Antenatal clinic between June 2013 and January 2015 were approached to participate in the study. Women aged between 18-40 years, with a singleton pregnancy and who could speak/understand English were eligible for inclusion. All women in this study received routine antenatal clinical care at the Obstetric Clinic at Monash Medical Centre. Women were seen by a team of obstetricians and midwives throughout pregnancy.

5.4.1.1 Diagnosis of gestational diabetes

All women attending maternity services within Monash Health are recommended to have a random blood glucose test at their first booking visit (approximately 13 weeks gestation) as part of routine

antenatal testing. Women with a high random blood glucose (> 7.0 mmol/l) then proceed to an OGTT. Women deemed at high risk for gestational diabetes or with previous gestational diabetes are recommended to undergo an OGTT at first booking visit. An OGTT is performed following an overnight fast and involves a 75 g liquid glucose load and blood sampling at 0 (fasting), 60 and 120 minutes.

The diagnostic criteria used by Monash Health to diagnose gestational diabetes are presented in **Table 5.1**. If the OGTT is abnormal in early pregnancy (fasting glucose ≥ 5.5 mmol/l and/or 2 hour glucose ≥ 8.0 mmol/l), women are referred to the Antenatal Diabetes Service and diagnosed with early gestational diabetes. It is important to note that the criteria used to diagnose gestational diabetes at Monash Health is different to the newly revised recommendations of ADIPS (**Section 1.1.2**) (ADIPS, 2014). The Monash Health procedure is currently under review and is expected to follow ADIPS guidelines in the imminent future.

Table 5.1 Guidelines for the diagnosis of gestational diabetes

	Fasting	1 hour	2 hour
Monash Health	≥ 5.5 mmol/l	N/A	≥ 8.0 mmol/l
ADIPS (ADIPS, 2014)	≥ 5.1 mmol/l	≥ 10.0 mmol/l	≥ 8.5 mmol/l

*One abnormal glucose value sufficient for diagnosis.

All women with normal early pregnancy glucose testing are recommended to have an OGTT between 24-28 weeks gestation. Women are diagnosed with gestational diabetes if the 24-28 weeks OGTT is abnormal, as outlined in **Table 5.1**, and referred to the Antenatal Diabetes Service. Women with normal OGTT values at 24-28 weeks gestation are considered to be a non-diabetic pregnancy.

5.4.1.2 Treatment guidelines for women diagnosed with gestational diabetes

Women referred to the Antenatal Diabetes Service attend a lifestyle education session. These sessions are co-ordinated by Monash Health diabetes educators and endocrinologists and advise women on diet and exercise. They also provide information on home blood glucose monitoring to be performed on three to four occasions per day. Women attended frequent clinic review with a diabetes physician and were typically seen every 1-2 weeks until birth, dependent on clinical indication. If more than one in three home capillary blood glucose readings were > 7.0 mmol/l postprandially or > 5.5 mmol/l fasting, insulin therapy was commenced. A full description of the Antenatal Diabetes Service at Monash Medical Centre is described in Stewart et al. (2012).

5.4.1.3 Incidence of gestational diabetes at Monash Medical Centre

In 2014, 10.6% of pregnant women attending Monash Medical Centre had gestational diabetes (personal communication with Bethany Carr, research midwife at Monash Medical Centre). Of these, 35% received insulin in pregnancy and 65% were treated by modifications to diet and exercise.

5.4.2 Maternal characteristics

Consent was obtained to access data on maternal characteristics. Data were extracted from the Monash Health Birthing Outcomes System (BOS) database, a computerised data collection system. Midwives caring for women enter pregnancy and birth outcome data into BOS. Maternal characteristics accessed for this study included demographic data (age and country of birth), pregnancy related data (weight, height, BMI, gravida, parity), lifestyle factors (alcohol and smoking status), pre-existing medical conditions and current obstetric conditions. Agreed expected delivery dates (based on last menstrual period and/or first trimester ultrasound) were also obtained from BOS. Note that data on ethnicity is not commonly collected in Australia and self-reported country of birth (grouped into regions) is used instead as a proxy measure for ethnicity (Hilder et al., 2014). Women were grouped into four ethnic categories: Caucasian (born in Australia, New Zealand, the United Kingdom or South Africa), South Asian (born in India, Pakistan, Bangladesh or Sri Lanka), South East/East Asian (born in China, Japan, Nepal, Vietnam, Thailand or Malaysia) and Other (born in Northern Africa, Middle East or South America).

Maternal data regarding glucose levels at booking and screening as well as self-monitored glucose levels in women diagnosed with gestational diabetes were collected from scanned antenatal medical records. HbA1c is also measured in antenatal serum testing in early pregnancy and was obtained from scanned medical records. HbA1c is formed by the non-enzymatic catalysis of haemoglobin A and glucose, a relatively irreversible process. HbA1c is positively related to the glucose concentration of red blood cells and can reflect the average blood glucose level within the past 8 to 10 weeks (Yu et al., 2014). Unfortunately, gestational weight gain was poorly recorded in women undergoing obstetric care at Monash Medical Centre and is not included in the present analysis.

In the present study, glycaemic control was determined by expert opinion. Associate Professor Carolyn Allan, an endocrinologist at Monash Medical Centre, classified gestational diabetic women with good, reasonable, fair or poor glycaemic control. Women were defined with good glycaemic control if the majority of home glucose readings were at target, with reasonable control if two out of three glucose readings were at target, and with fair glycaemic control if less than two out of three glucose readings were within target. If all home glucose readings were not at target women were defined with poor glycaemic control.

Women diagnosed with gestational diabetes have an increased risk of recurrence of hyperglycaemia in subsequent pregnancies and a risk of developing Type 2 diabetes in later life (ADIPS, 2014). Subsequently, women are recommended to have an OGTT 6-12 weeks post-partum and are diagnosed with diabetes based on guidelines used to diagnose Type 2 diabetes in the general population outside

of pregnancy (RACGP, 2014). Women were diagnosed with pre-diabetes if fasting glucose was 6.1-6.9 mmol/l or 2 hour glucose was 7.8-11.0 mmol/l. Women were diagnosed with diabetes if fasting glucose was > 6.9 mmol/l or 2 hour glucose was > 11.0 mmol/l. Postnatal OGTT data were obtained from scanned medical records.

5.4.3 Ultrasound measurements

Obstetric ultrasounds were performed between 32^{+0} and 33^{+6} weeks of gestation. High resolution ultrasound imaging was performed by trained professional staff at Diagnostic Imaging and Fetal Monitoring Services at Monash Medical Centre. Ultrasound examinations were performed on a Philips IU22 xMatrix ultrasound system, using broad band linear and micro-convex transducers with frequency range from 5-17 mHz providing sub-millimetre resolution.

5.4.3.1 Fetal biometry

Parameters of fetal biometry, including head circumference (HC), bi-parietal diameter (BPD), abdominal circumference (AC) and femur length (FL), were measured using a trans-abdominal probe (Nisbet et al., 2002). HC and BPD were measured in the transverse plane of the fetal head, including the falx cerebri anteriorly and posteriorly, and the cavity of the septum pellucidum and the thalamus in the midline. The BPD was measured from the outer edge of the nearer parietal bone to the inner edge of the distant parietal bone. An ellipse was drawn around the outer skull to measure HC. AC was measured in the transverse plane at the level of the liver and stomach, including the left portal vein and spine. FL was measured perpendicular to the beam with the full length of the bone in view. These measurements are illustrated in **Figure 5.1**.

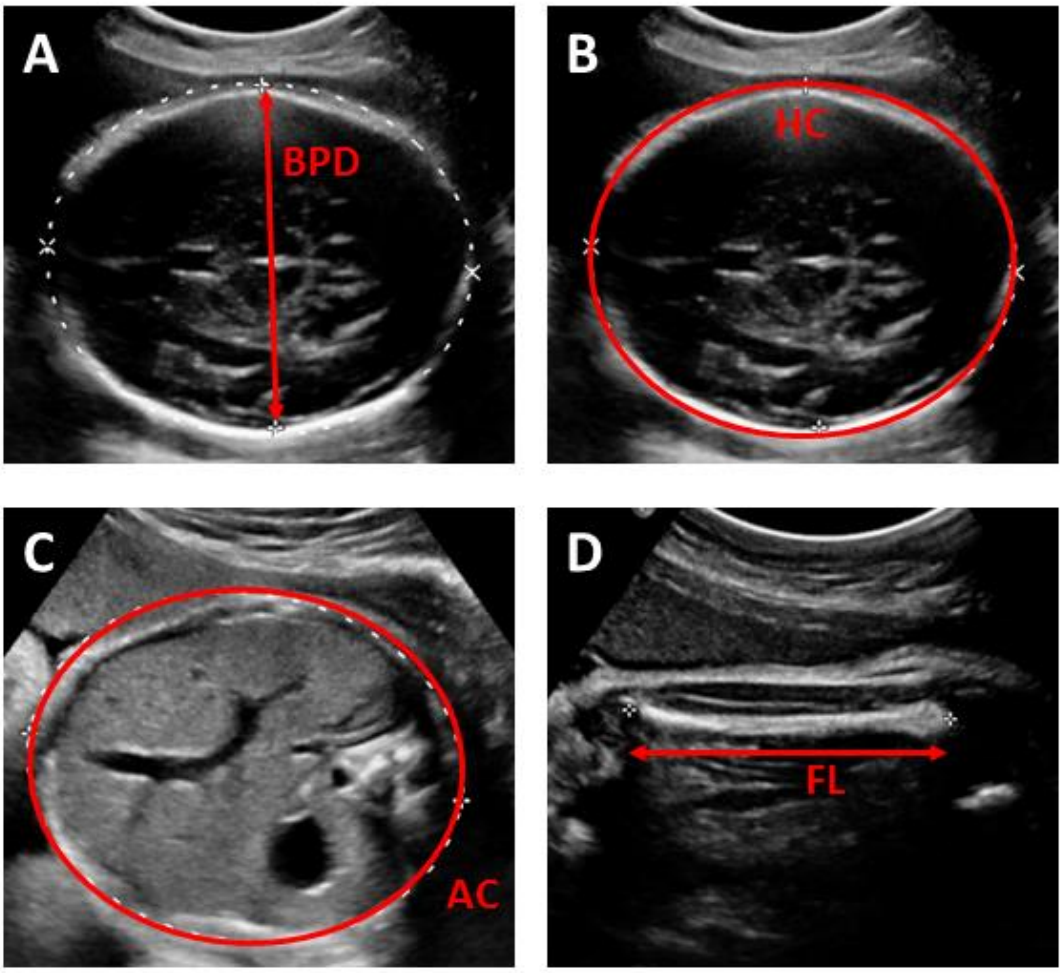

Figure 5.1 Measuring fetal biometry parameters by obstetric ultrasound at 32-34 weeks gestation
A Bi-parietal diameter (BPD), **B** head circumference (HC), **C** abdominal circumference (AC) and **D** femur length (FL).

EFW was derived using the Hadlock IV formula Hadlock et al. (1985). This is considered among the most accurate formulae for estimating fetal weight and is the most widely used in Australian practice. The Hadlock IV formula integrates measurements of BPD, HC, AC and FL to estimate fetal weight and is shown below:

$$\text{Log}_{10}\,\text{EFW} = 0.3596 + (0.00061 \times \text{BPD} \times \text{AC}) + (0.042 \times \text{AC}) + (0.174 \times \text{FL}) + (0.0064 \times \text{HC}) - (0.00386 \times \text{AC} \times \text{FL})$$

EFW percentiles were also determined. EFWs were charted against population standards to determine the corresponding percentile band. Fetal growth charts used within Monash Health were originally developed by Hadlock et al. (1991), adapt the notion of proportionality proposed by Gardosi et al. (1992), and are derived from local populations as proposed by Mikolajczyk et al. (2011). Weight

percentile charts used at Monash Health incorporate the mean birth weight at Monash Health at 40 weeks gestation (3464 g).

5.4.3.2 Umbilical and fetal blood flow profiles

Umbilical artery and middle cerebral artery pulsatility indices were measured at 32-34 weeks gestation (**Figure 5.2**). Umbilical artery flow velocity waveforms were measured by Doppler ultrasound in a loop of the umbilical cord. Abnormal umbilical artery blood flow is a marker of uteroplacental insufficiency, with raised pulsatility index values indicative of increased placental resistance. Middle cerebral artery blood flow was assessed by colour Doppler ultrasound. A low middle cerebral artery pulsatility index reflects the redistribution of cardiac output to the brain, indicating fetal brain sparing.

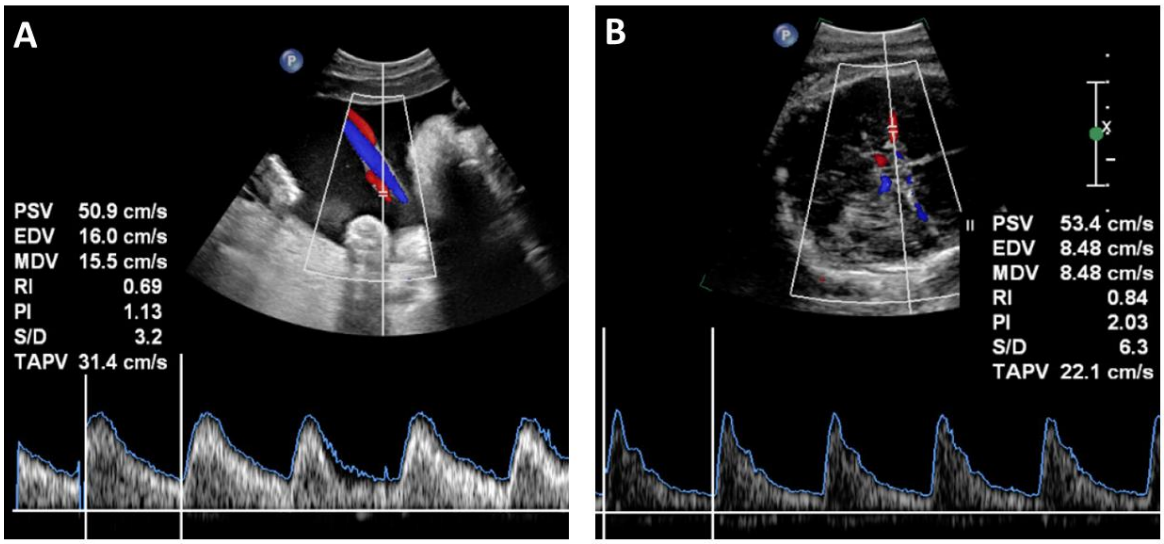

Figure 5.2 Measuring umbilical artery pulsatility index and middle cerebral artery pulsatility index at 32-34 weeks gestation

A Umbilical artery PI and **B** middle cerebral artery PI measured by Doppler ultrasound. Red and blue indicates blood flow toward and away from the ultrasound transducer.

5.4.3.3 Amniotic fluid index

Amniotic fluid index (AFI), as a measure of amniotic fluid volume, was assessed by ultrasound at 32-34 weeks gestation. AFI was calculated as the sum of the single deepest vertical pocket measurements recorded in each quadrant (**Figure 5.3**). AFI values between 5-25 cm are considered normal. AFI provides information about fetal renal function, as amniotic fluid volume predominantly reflects fetal urination at this gestational age (Gilbert and Brace, 1993).

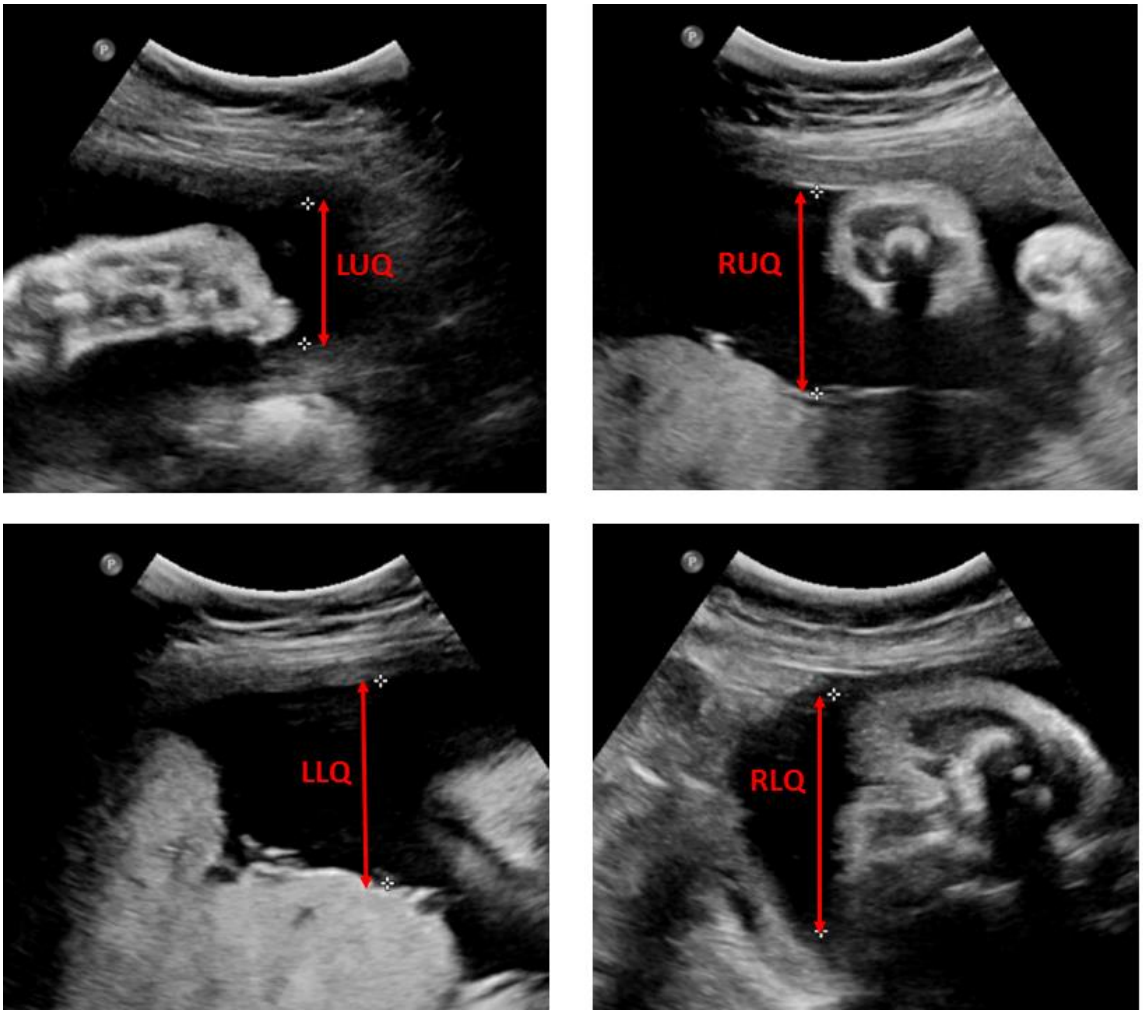

Figure 5.3 Measuring amniotic fluid index at 32-34 weeks gestation
AFI is determined by summing the single deepest pocket measurements of amniotic fluid in each quadrant. LUQ=left upper quadrant, RUQ=right upper quadrant, LLQ=left lower quadrant, RLQ=right lower quadrant.

5.4.3.4 Kidney measurements

Fetal kidney size, as a measure of renal sufficiency, was assessed by obstetric ultrasound. Fetal kidney length, width and depth were measured using a trans-abdominal probe. Kidney length was measured in the sagittal plane (**Figure 5.4A**), with the maximum longitudinal length measured by placing the callipers on the outer edges of the caudal and cranial side. Width (transverse kidney diameter) and depth (antero-posterior kidney diameter) were measured perpendicular to each other in the transverse plane (**Figure 5.4B**). Left and right kidney measurements were recorded. In four participants, a single kidney was measured due to unfavourable fetal position or maternal habitus.

The most common and widely accepted method to calculate fetal kidney volume by ultrasound is the prolate (elongated) ellipsoid formula (Gloor et al., 1997, Chitty and Altman, 2003, Verburg et al., 2007,

Geelhoed et al., 2009, van Vuuren et al., 2012), as first described by Jeanty et al. (1982). Fetal kidney volume was calculated by the approximation of a prolate ellipsoid, where:

$$\text{Volume}_{\text{ellipsoid}} = \text{length x width x depth x } 0.523$$

Previous publications assessing fetal kidney size by ultrasound report mean kidney dimensions or mean kidney volume (Cohen et al., 1991, Konje et al., 1996, Gloor et al., 1997, Konje et al., 1997), combined kidney volume (Verburg et al., 2007) or left and right kidney volumes (Neves et al., 2013). Each of these parameters will be reported in this study. Data will be presented as length, width, depth and volume for left and right kidneys. Mean fetal kidney volume (average of left volume and right volume), total fetal kidney volume (left volume and right volume combined; excluding fetuses where only one kidney was measured), and kidney dimensions relative to body weight will also be presented. Maximum renal pelvis diameters of left and right kidneys were also measured (**Figure 5.4C**).

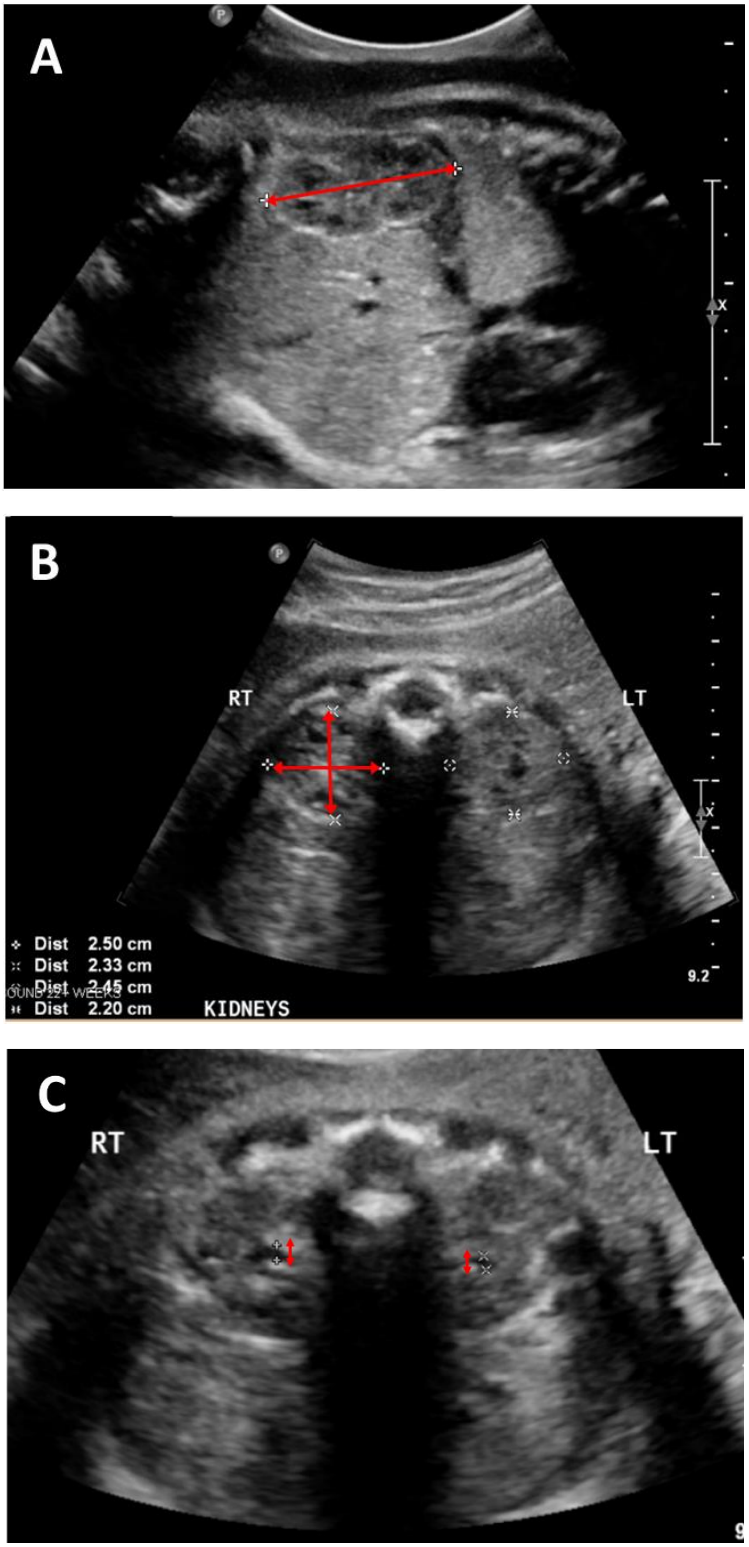

Figure 5.4 Fetal kidney size measured by obstetric ultrasound at 32-34 weeks gestation
A Kidney length, **B** kidney width (transverse kidney diameter) and depth (antero-posterior kidney diameter) and **C** maximum renal pelvis diameter.

5.4.3.5 Intra-observer and inter-observer variability

The intra-observer variability (coefficient of variation) for Doppler ultrasound measurements was 7.3%. The intra-observer variability for kidney measurements by ultrasound imaging was 4.4%. Unfortunately, the Fetal Monitoring Unit at Monash Medical Centre relies on a single sonographer at any one time to perform ultrasound scans so inter-observer variability measures were not obtainable.

5.4.4 Birth data

Consent was obtained to access newborn data from BOS. Data including length of gestation, mode of delivery, umbilical cord lactate values, Apgar scores, birth weight, birth head circumference, birth length, ponderal index (birth weight in g/(length in cm)^3 x 100) and baby sex were obtained. Admission to special care nursery and neonatal complications will also be described. Placental weight is not recorded at delivery at Monash Health.

5.4.5 Additional data

Fetal morphology and anomaly scans are typically performed between 19-22 weeks gestation as part of routine antenatal care. These ultrasounds were undertaken at various medical imaging centres across Melbourne. Data regarding fetal biometry from second trimester ultrasounds were collected from antenatal records.

5.4.6 Statistical analysis

The data presented in this chapter are preliminary and obtained as part of an ongoing study at Monash Medical Centre. The governing ethics committees have approved 85 women to be recruited per group. The low numbers of women recruited to date with Type 1 diabetes, Type 2 diabetes and early gestational diabetes prevent an informative quantitative analysis so data from these groups is not presented. Data are presented from 36 women with gestational diabetes in pregnancy and 42 women with normal glucose in pregnancy. Optimal sample size was calculated prior to recruitment to ensure adequate power to detect statistical significance. A 15% difference in kidney size between diabetic and non-diabetic populations would be of biological significance. Previous studies report a 30% standard deviation for kidney size (Verburg et al., 2007). Based on these values and a statistical power of 0.8, a sample size of 32 subjects per group is required for a level of significance of 0.05. Note, ethics is approved for a total of 85 subjects per group to allow for sub-analysis at the completion of the study.

Data were analysed using STATA and SPSS software. Data were graphically presented using GraphPad Prism software. Data were tested for normality using a D'Agostino-Pearson omnibus normality test. Maternal and fetal parameters in non-diabetic and gestational diabetic pregnancies were analysed by Wilcoxon rank sum test. OGTT results and fetal growth trajectories were analysed using repeated

measures ANOVA. A glucose AUC value of the OGTT was calculated using GraphPad. Correlations between fetal and maternal variables were assessed by Spearman's rank co-efficient with Bonferroni correction using STATA. A population proportions test was used to analyse the frequency of maternal country of birth, pre-existing medical complications, obstetric complications and birth outcomes. Data are presented as mean ± SD or median ± inter-quartile range (IQR). $P<0.05$ was considered statistically significant.

5.5 Results

5.5.1 Maternal parameters

5.5.1.1 Age, weight and parity are similar in women with and without gestational diabetes

Maternal characteristics of women without diabetes in pregnancy and women with gestational diabetes are presented in **Table 5.2**. There was no difference in the age of women at conception or the gestational age at antenatal booking appointment. Maternal weight, height and BMI were comparable between women with gestational diabetes and women without diabetes in pregnancy. Gravida and parity were also similar between groups. 40% of women without diabetes and 53% of women with gestational diabetes were nulliparous.

Maternal country of birth is presented in **Table 5.2**. There was a greater proportion of women of Caucasian ethnicity without diabetes (*P*=0.031). Women born in South East/East Asia were equally represented across the two groups. There were twice as many women born in South Asia with gestational diabetes than without diabetes yet this was not of statistical significance (*P*=0.326). In a larger study population the difference in the proportion of South-Asian women with and without gestational diabetes would be expected to reach statistical significance.

Table 5.2 Maternal characteristics

		No diabetes (*n*=42)	**Gestational diabetes** (*n*=36)	***P***
Age at conception, years		31.3 ± 4.8	31.8 ± 4.8	0.649
Weight at booking, kg		67.2 ± 13.9	66.4 ± 19.4	0.470
Height at booking, cm		163.3 ± 6.0	161.3 ± 6.5	0.172
BMI at booking, kg/m^2		24.5 ± 4.5	25.0 ± 6.7	0.744
Gestation at booking, weeks		14.2 ± 4.1	14.4 ± 5.0	0.750
Gravida		2.3 ± 1.2	2.1 ± 1.2	0.337
Parity		0.8 ± 0.9	0.6 ± 0.9	0.198
Country of birth, *n*(%)	**Caucasian**	22 (52%)	9 (25%)	**0.031**
	South East/East Asian	12 (29%)	11 (31%)	0.833
	South Asian	6 (14%)	13 (36%)	0.326
	Other	2 (5%)	3 (8%)	0.896

Data regarding maternal country of birth analysed by population proportions test. All other data analysed by Wilcoxon rank sum test and presented as mean ± SD.

5.5.1.2 Elevated early pregnancy HbA1c in women with gestational diabetes

As part of routine antenatal serum testing, it is recommended that all women have a random blood glucose and HbA1c test in early pregnancy (approximately 13 weeks gestation) (Monash Health Procedure, 2014). Early random blood glucose tests were performed in 71% of non-diabetic women and in 64% of women later identified with gestational diabetes. 71% of non-diabetic women had an

early pregnancy HbA1c measured, yet only 36% of women with gestational diabetes had an HbA1c test in early pregnancy. There was no difference in random blood glucose between women without diabetes in late gestation or women with gestational diabetes in late gestation (no diabetes 4.9 ± 1.0 mmol/l, gestational diabetes 5.0 ± 1.1 mmol/l; *P*=0.359). Early pregnancy HbA1c values were elevated by 8% in women who were later diagnosed with gestational diabetes (no diabetes 32.8 ± 2.7 mmol/mol, gestational diabetes 35.4 ± 2.16 mmol/mol; *P*=0.005; **Figure 5.5**).

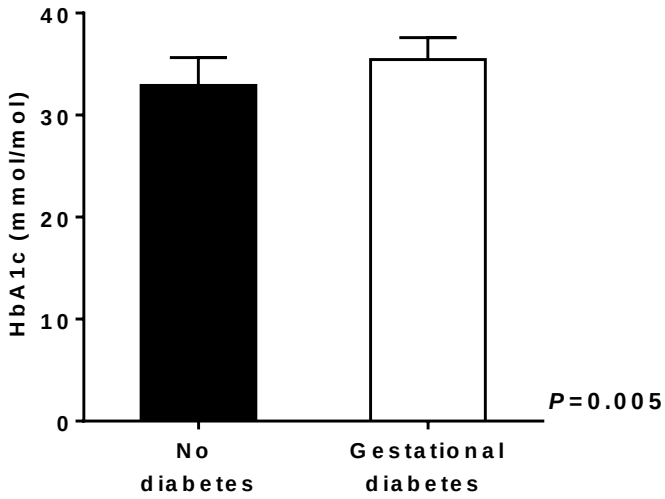

Figure 5.5 HbA1c in early pregnancy
Glycated haemoglobin measured in early pregnancy in women who would later be identified without diabetes or with gestational diabetes in the third trimester. Data analysed by Wilcoxon rank sum test. Values are mean ± SD. *n*(no diabetes)=30, *n*(gestational diabetes)=13.

5.5.1.3 Oral glucose tolerance test as routine testing for gestational diabetes

All women had the recommended OGTT as part of routine screening for gestational diabetes. The OGTT was performed at a similar gestational age (no diabetes 26.7 ± 1.7 weeks, gestational diabetes 26.4 ± 1.4 weeks; *P*=0.184).

Women diagnosed with gestational diabetes had an 11% higher fasting blood glucose concentration (no diabetes 4.3 ± 0.3 mmol/l, gestational diabetes 4.8 ± 0.7 mmol/l; *P*=0.001). It should be noted that the mean fasting blood glucose concentration in women with gestational diabetes was lower than the diagnostic criteria for gestational diabetes based on fasting glucose (**Table 5.1**). Furthermore, only 17% of women diagnosed with gestational diabetes had an elevated fasting glucose level (≥ 5.5 mmol/l based on Monash Health diagnostic criteria). If the ADIPS criteria were followed (fasting glucose ≥ 5.1 mmol/l), then 36% of women with gestational diabetes would have been diagnosed. Note that no

women in the control group would have been diagnosed with gestational diabetes if the ADIPS criteria were followed.

Women diagnosed with gestational diabetes had a 37% higher blood glucose concentration at 60 minutes (no diabetes 7.0 ± 1.2 mmol/l, gestational diabetes 9.6 ± 2.0 mmol/l; *P*<0.0001) and a 49% higher blood glucose concentration at 120 minutes (no diabetes 5.9 ± 1.1 mmol/l, gestational diabetes 8.8 ± 1.4 mmol/l; *P*<0.0001) (**Figure 5.6**).

Values from the OGTT were converted to a glucose AUC. Glucose AUC was 36% greater in women diagnosed with gestational diabetes than in non-diabetic women (no diabetes 724.9 ± 94.3 mmol/l.min, gestational diabetes 984.5 ± 162.8 mmol/l.min; *P*<0.0001; **Figure 5.7**).

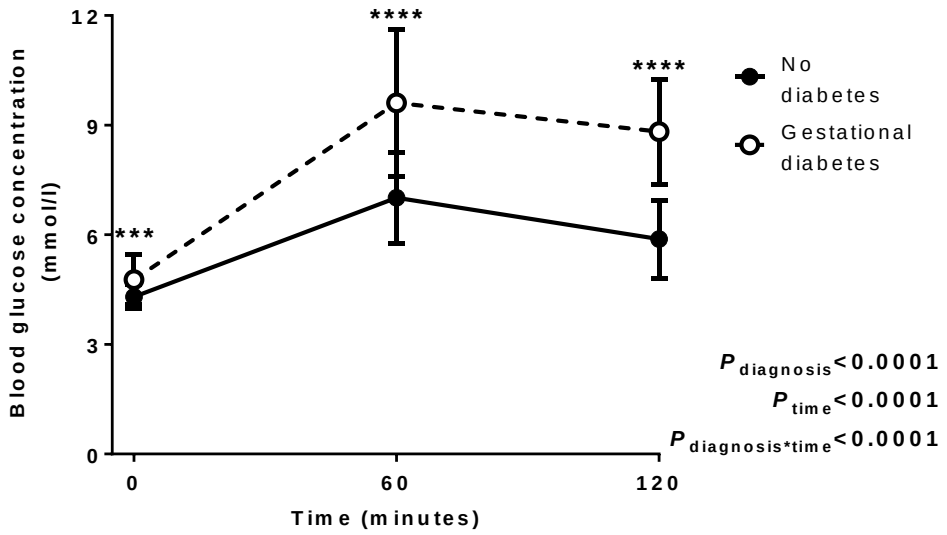

Figure 5.6 Oral glucose tolerance test to diagnose gestational diabetes
OGTT results for women without diabetes and women diagnosed with gestational diabetes. Data analysed by repeated measures ANOVA. Values are mean ± SD. *n*(no diabetes)=42, *n*(gestational diabetes)=36. ***P<0.001, ****P<0.0001 refer to differences between the two groups at the three time points.

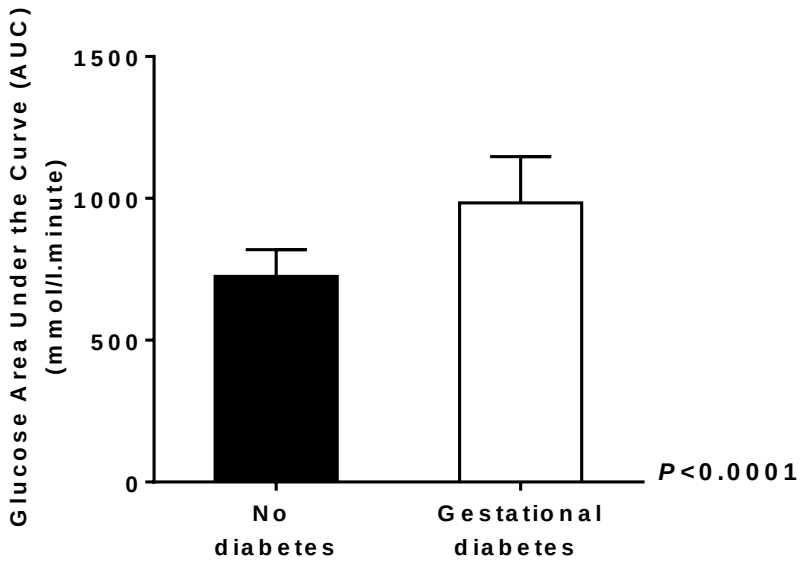

Figure 5.7 Glucose Area Under the Curve
Glucose AUC of women without diabetes and women diagnosed with gestational diabetes. Data analysed by Wilcoxon rank sum test. Values are mean ± SD. *n*(no diabetes)=42, *n*(gestational diabetes)=36.

5.5.1.4 Associations between maternal characteristics and glucose profiles in pregnancy

Spearman's correlation analyses were performed to assess associations between maternal parameters and glucose in pregnancy (see **Table 5.3**).). Random blood glucose concentrations measured in early pregnancy were positively correlated with maternal weight and BMI at booking appointment, and with 1 and 2 hour glucose levels during the OGTT. Maternal HbA1c measured in early pregnancy correlated positively with all OGTT values. Fasting glucose measured at the OGTT correlated with maternal BMI at booking appointment and with other OGTT values. Maternal age at conception did not correlate with any maternal variable.

Table 5.3 Spearman's rank correlation coefficients for associations between maternal characteristics and glucose in pregnancy

		Random glucose	HbA1c	Fasting glucose	1 hour glucose	2 hour glucose	Glucose AUC	Body weight at booking	BMI at booking
HbA1c at booking	**rho**	0.319							
	P	0.055							
Fasting glucose OGTT	**rho**	0.108	**0.509**						
	P	0.454	**0.0008**						
1 hour glucose OGTT	**rho**	**0.287**	**0.448**	**0.428**					
	P	**0.043**	**0.004**	**0.0001**					
2 hour glucose OGTT	**rho**	**0.353**	**0.449**	**0.414**	**0.685**				
	P	**0.012**	**0.004**	**0.0002**	**<0.0001**				
Glucose AUC	**rho**	**0.314**	**0.510**	**0.508**	**0.954**	**0.855**			
	P	**0.026**	**0.0008**	**<0.0001**	**<0.0001**	**<0.0001**			
Body weight at booking	**rho**	**0.293**	0.186	0.223	0.106	0.067	0.095		
	P	**0.033**	0.232	0.054	0.368	0.566	0.416		
BMI at booking	**rho**	**0.306**	0.161	**0.298**	0.078	0.125	0.106	**0.905**	
	P	**0.026**	0.303	**0.009**	0.508	0.285	0.367	**<0.0001**	
Age at conception	**rho**	-0.202	0.137	0.016	0.194	0.166	0.191	-0.062	-0.117
	P	0.146	0.380	0.894	0.095	0.154	0.101	0.590	0.307

Spearman's rho (top value), *P* value (bottom value). Negative values indicate a negative association. Values in bold are statistically significant.

5.5.1.5 One third of women with gestational diabetes required insulin therapy

Insulin therapy was required to treat 34% of women diagnosed with gestational diabetes. The majority of women started insulin therapy at 30-32 weeks gestation. There was no difference in maternal characteristics between women with gestational diabetes treated with insulin and women treated by modifications to diet and exercise alone (data not presented).

5.5.1.6 Women with gestational diabetes demonstrated good glycaemic control

The majority of women with gestational diabetes were defined with good glycaemic control (77%). Seventeen percent of women demonstrated reasonable glycaemic control and 6% had fair glycaemic control. No women were identified with poor glycaemic control in this study.

5.5.1.7 Similar prevalence of pre-existing medical conditions and current obstetric conditions

The frequencies of substance use, pre-existing medical conditions and current obstetric conditions are presented in **Table 5.4**. There was no statistical difference in the prevalence of vitamin D deficiency, anaemia or previous gestational diabetes between women with gestational diabetes and women without diabetes. Due to insufficient observations, statistical analysis of the prevalence of substance use, current obstetric conditions and other pre-existing medical conditions was not performed.

Table 5.4 Substance use, pre-existing medical conditions and current obstetric conditions

	No diabetes (*n*=42; %)	**Gestational diabetes** (*n*=36; %)	***P***
Smoker > 20 weeks	1 (2%)	2 (6%)	N/A
Alcohol consumption > 20 weeks	0	1 (3%)	N/A
Pre-existing hypertension	0	1 (3%)	N/A
Hypothyroidism	2 (5%)	1 (3%)	N/A
Low Vitamin D	33 (79%)	24 (67%)	0.314
Anaemia	2 (5%)	3 (8%)	0.880
Previous gestational diabetes	2 (5%)	4 (11%)	0.802
Previous macrosomia (≥ 4500g)	0	2 (6%)	N/A
Gestational thrombocytopenia	1 (2%)	1 (3%)	N/A
Pre-eclampsia	1 (2%)	2 (6%)	N/A

Analysed by population proportions test. N/A indicates insufficient observations to perform statistical analysis.

5.5.1.8 Post-partum oral glucose tolerance test in women with gestational diabetes

81% of women diagnosed with gestational diabetes underwent a post-partum OGTT. Of these, 21% (6 out of 29 women) had elevated glucose levels indicating pre-diabetes. No women were diagnosed with overt diabetes at 6-12 weeks post-partum.

5.5.2 Fetal biometry parameters

5.5.2.1 Normal fetal biometry at 19-22 weeks gestation

Data from second trimester fetal anomaly ultrasounds performed between 19-22 weeks gestation were obtained from medical records. Data are presented in **Table 5.5**. Ultrasounds were performed at a similar gestational age. There was no difference in bi-parietal diameter, head circumference, abdominal circumference or femur length between women who would and would not be diagnosed with gestational diabetes in third trimester.

EFW was also similar between fetuses of women who would be later diagnosed with gestational diabetes and those who would not have diabetes (no diabetes 363.1 ± 74.2 g, gestational diabetes 348.5 ± 49.0 g; *P*=0.413; **Figure 5.8**). EFW percentiles were also similar in the two groups (*P*=0.941, **Figure 5.9**). There was no difference in EFW between male and female fetuses at 19-22 weeks gestation (*P*=0.302).

Table 5.5 Fetal biometry at 19-22 weeks gestation

	No diabetes (*n*=34)	**Gestational diabetes** (*n*=28)	***P***
Gestational age at ultrasound, weeks	20.3 ± 0.9	20.2 ± 0.7	0.770
Bi-parietal diameter, mm	47.8 ± 3.5	47.8 ± 2.9	0.914
Head circumference, mm	177.6 ± 12.7	176.1 ± 9.5	0.751
Abdominal circumference, mm	156.4 ± 15.5	152.3 ± 9.3	0.121
Femur length, mm	33.4 ± 3.8	32.8 ± 2.3	0.995

Data analysed by Wilcoxon rank sum test. Values are mean ± SD.

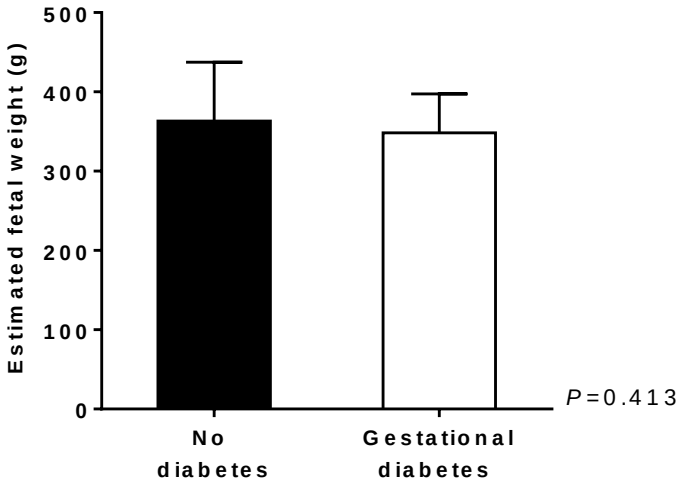

Figure 5.8 Estimated fetal weight at 19-22 weeks gestation

EFW at 19-22 weeks gestation for women without diabetes and women with subsequent gestational diabetes. Data analysed by Wilcoxon rank sum test. Values are mean ± SD. *n*(no diabetes)=34, *n*(gestational diabetes)=29.

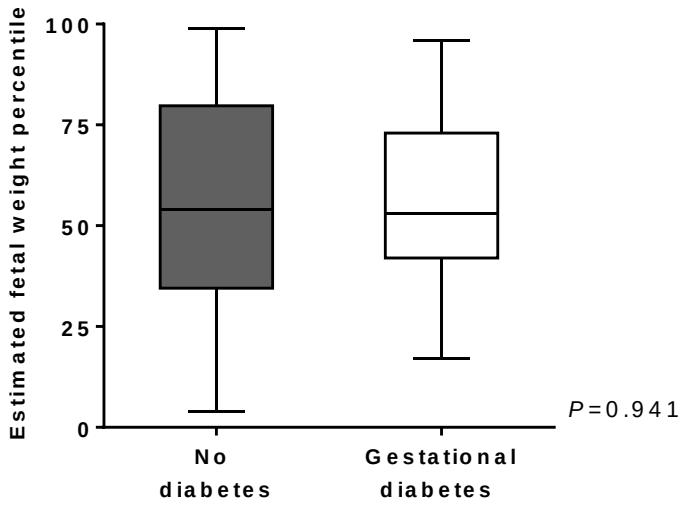

Figure 5.9 Estimated fetal weight percentile at 19-22 weeks gestation
EFW percentile at 19-22 weeks gestation for women without diabetes and women with subsequent gestational diabetes. Data analysed by Wilcoxon rank sum test. Data presented as box and whisker plot. *n*(no diabetes)=34, *n*(gestational diabetes)=29.

5.5.2.2 Lower abdominal circumference and EFW at 32-34 weeks gestation in gestational diabetes

All women underwent a third trimester fetal biometry scan between 32 and 34 weeks gestation. Parameters of fetal biometry are presented in **Table 5.6**. Ultrasounds were performed at a similar gestational age (*P*=0.427). There was a trend for the bi-parietal diameter to be reduced in fetuses of women with gestational diabetes (*P*=0.081). There was no difference in head circumference or femur length between groups. However, abdominal circumference was 4% smaller in fetuses of women with gestational diabetes than in fetuses of women without diabetes (*P*=0.002).

EFW was 7% lower in fetuses of women with gestational diabetes (no diabetes 2204.8 ± 239.0 g, gestational diabetes 2052.1 ± 264.7 g; *P*=0.006; **Figure 5.10**). EFW percentiles were also lower in women diagnosed with gestational diabetes (P=0.002, **Figure 5.11**).

Table 5.6 Fetal biometry at 32-34 weeks gestation

	No diabetes (*n*=42)	**Gestational diabetes** (*n*=36)	***P***
Gestational age at ultrasound, weeks	32.9 ± 0.6	32.8 ± 0.8	0.427
Bi-parietal diameter, mm	84.0 ± 3.2	82.7 ± 3.4	0.081
Head circumference, mm	302.1 ± 8.5	298.3 ± 19.5	0.634
Abdominal circumference, mm	298.2 ± 13.5	286.8 ± 16.9	**0.002**
Femur length, mm	63.2 ± 2.8	62.6 ± 2.3	0.305

Data analysed by Wilcoxon rank sum test. Values are mean ± SD.

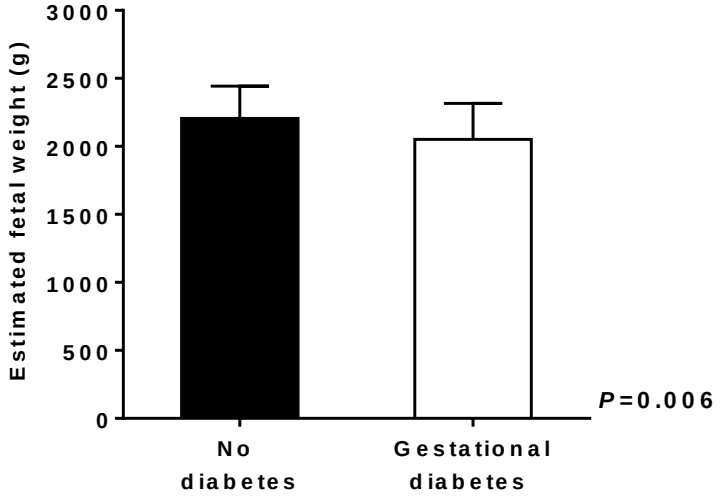

Figure 5.10 Estimated fetal weight at 32-34 weeks gestation
EFW at 32-34 weeks gestation for women without diabetes and women with gestational diabetes. Data analysed by Wilcoxon rank sum test. Values are mean ± SD. *n*(no diabetes)=42, *n*(gestational diabetes)=36.

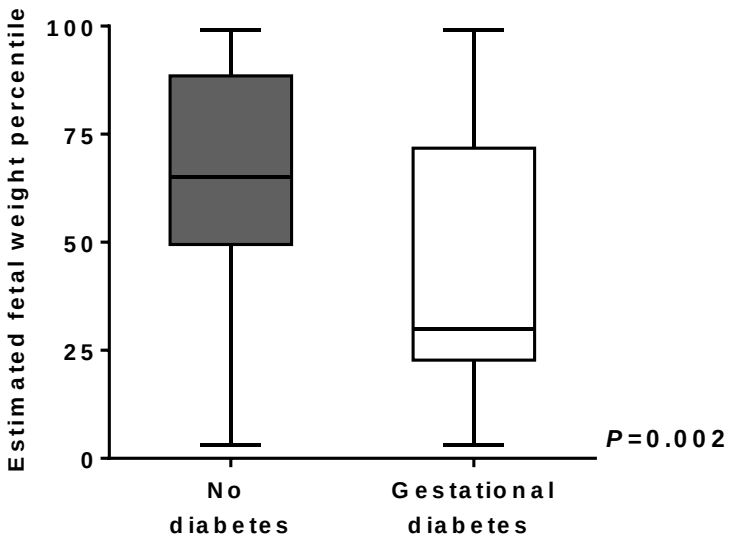

Figure 5.11 Estimated fetal weight percentile at 32-34 weeks gestation
EFW percentile at 32-34 weeks gestation for fetuses of women without diabetes and women with gestational diabetes. Data analysed by Wilcoxon rank sum test. Data presented as box and whisker plot. *n*(no diabetes)=42, *n*(gestational diabetes)=36.

Interestingly, the effect of gestational diabetes on fetal biometry at 32-34 weeks gestation was more pronounced in women requiring insulin therapy. While the number of women treated with insulin was low (*n*=12), abdominal circumference was significantly reduced in women requiring insulin therapy compared with women treated with diet and exercise (diet and exercise 293.1 ± 15.2 mm, insulin 276.3

± 15.7 mm; *P*=0.015; **Figure 5.12A**). EFW was also lower in women treated with insulin (diet and exercise 293.1 ± 15.2 mm, insulin 276.3 ± 15.7 mm; *P*=0.048; **Figure 5.12B**).

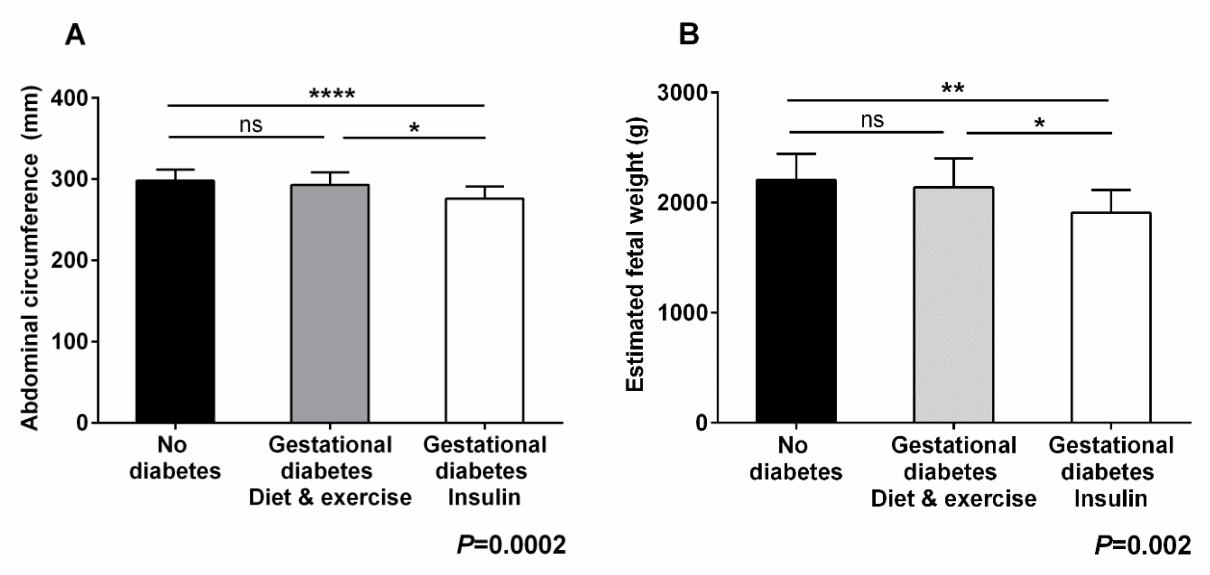

Figure 5.12 Abdominal circumference and estimated fetal weight at 32-34 weeks gestation in women treated with and without insulin

A Fetal abdominal circumference and **B** EFW at 32-34 weeks gestation in women without diabetes, women with gestational diabetes treated with modifications to diet and exercise, and women with gestational diabetes treated with insulin. Analysis by Kruskal Wallis one-way ANOVA and Dunn's multiple comparisons test. Values are mean ± SD. *n*(no diabetes)=42, *n*(gestational diabetes diet and exercise)=24, *n*(gestational diabetes insulin)=12. **P*<0.05, ***P*<0.01, *****P*<0.0001.

There was no difference in EFW between male and female fetuses at 32-34 weeks gestation (*P*=0.302). Male fetuses of women with gestational diabetes had lower EFW than male fetuses of women without diabetes (*P*=0.049) (**Table 5.7**). There was no statistical difference in EFW between female fetuses of women with and without gestational diabetes (**Table 5.7**).

Table 5.7 Male and female estimated fetal weight at 32-34 weeks gestation

	No diabetes (*n*)	**Gestational diabetes** (*n*)	***P***
Male EFW (g)	2240.6 ± 230.9 (25)	2036.0 ± 324.5 (16)	**0.049**
Female EFW (g)	2134.1 ± 258.3 (14)	2062.8 ± 203.1 (18)	0.999
P	0.999	0.998	

Data analysed by Kruskal Wallis one-way ANOVA and Dunn's multiple comparison test. Values are mean ± SD.

5.5.2.3 Lower amniotic fluid index and fetal middle cerebral artery pulsatility index in gestational diabetes

AFI was 8% lower in women with gestational diabetes (no diabetes 15.5 ± 3.5 cm, gestational diabetes 14.3 ± 3.9 cm; *P*=0.027; **Figure 5.13**). This reduction in AFI was more pronounced in women treated

with insulin than women treated with diet and exercise, although this was not of statistical significance (diet and exercise 14.5 ± 3.0 cm, insulin 12.6 ± 2.3 cm; *P*=0.282). It should be noted that while AFI was found to be reduced in gestational diabetes, all values were within the normal clinical range.

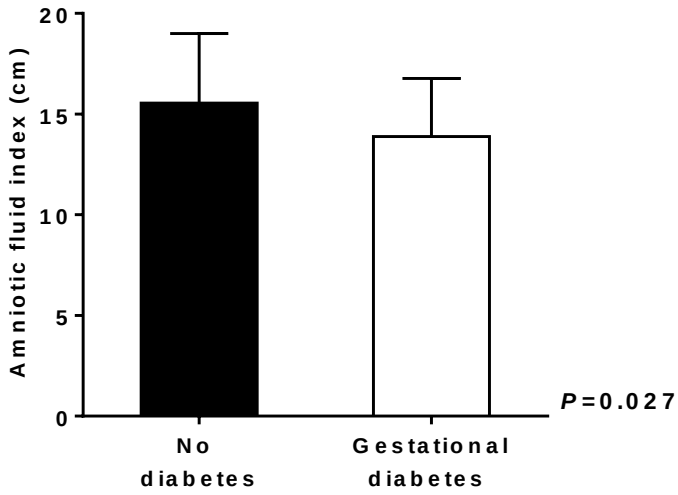

Figure 5.13 Amniotic fluid index at 32-34 weeks gestation
AFI at 32-34 weeks gestation in women without diabetes and women with gestational diabetes. Data analysed by Wilcoxon rank sum test. Values are mean ± SD. *n*(no diabetes)=41, *n*(gestational diabetes)=36.

The middle cerebral artery pulsatility index was 9% lower in fetuses of women with gestational diabetes than in fetuses of women without diabetes in pregnancy (no diabetes 2.17 ± 0.36, gestational diabetes 1.98 ± 0.30; *P*=0.012; **Figure 5.14**). In contrast, umbilical artery pulsatility index was similar in the two groups (no diabetes 1.02 ± 0.33, gestational diabetes 1.08 ± 0.46; *P*=0.939) and the ratio of middle cerebral artery: umbilical artery pulsatility indices was also similar in the two groups (no diabetes 2.21 ± 0.44, gestational diabetes 2.10 ± 0.40; *P*=0.274). It should be noted that while middle cerebral artery pulsatility index was reduced in gestational diabetes, all values were within the normal clinical range. There was no difference in Doppler values between women with gestational diabetes treated with diet and exercise, or with insulin.

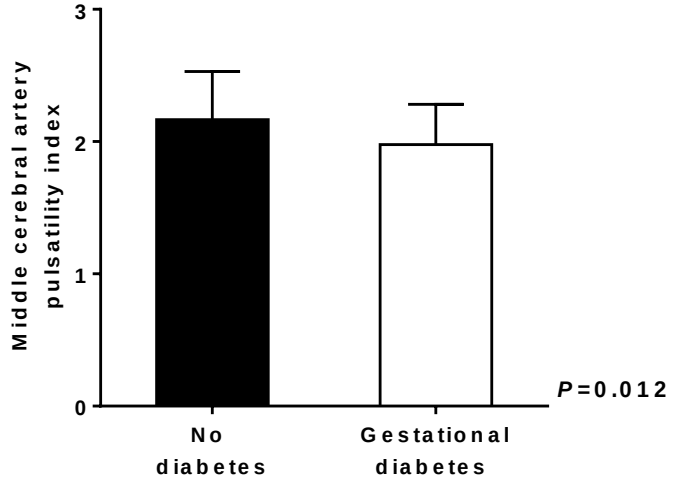

Figure 5.14 Fetal middle cerebral artery pulsatility index at 32-34 weeks gestation
Middle cerebral artery pulsatility index at 32-34 weeks gestation in women without diabetes and women with gestational diabetes. Data analysed by Wilcoxon rank sum test. Values are mean ± SD. *n*(no diabetes)=41, *n*(gestational diabetes)=34.

5.5.2.4 Correlation analyses between maternal characteristics and fetal biometry and wellbeing at 32-34 weeks gestation

Spearman's correlation analyses were performed to assess associations between maternal parameters and fetal growth in the third trimester (see **Table 5.8**). HbA1c in early pregnancy and fasting glucose at OGTT were not correlated with any parameter of fetal biometry. Two hour glucose and glucose AUC at OGTT were negatively correlated with abdominal circumference, EFW and middle cerebral artery pulsatility index. The association between glucose AUC at OGTT and EFW is presented in **Figure 5.15.** Maternal weight and maternal BMI at booking had a significant positive association with fetal biometry and EFW at 32-34 weeks gestation. As expected, EFW and fetal size had a positive association with gestational age at ultrasound.

No maternal parameters were associated with AFI, however AFI was positively associated with EFW (rho=0.323, *P*=0.004), abdominal circumference (rho=0.380, *P*=0.0006) and bi-parietal diameter (rho=0.321, *P*=0.005). Middle cerebral artery and umbilical artery pulsatility indices did not correlate with fetal biometry parameters.

Table 5.8 Spearman's rank correlation coefficients for associations between maternal characteristics and fetal growth at 32-34 weeks gestation

		HbA1c	Fasting glucose	1 hour glucose	2 hour glucose	Glucose AUC	Weight at booking	BMI at booking	Gestational age
Bi-parietal diameter	**rho**	-0.051	-0.036	-0.143	-0.154	-0.155	**0.273**	0.357	**0.272**
	P	0.744	0.756	0.222	0.186	0.184	**0.015**	0.113	**0.016**
Head circumference	**rho**	-0.009	-0.028	-0.065	-0.035	-0.044	**0.318**	**0.304**	**0.231**
	P	0.956	0.816	0.580	0.770	0.710	**0.005**	**0.035**	**0.042**
Abdominal circumference	**rho**	-0.274	-0.115	**-0.256**	**-0.306**	**-0.303**	**0.290**	**0.205**	**0.341**
	P	0.075	0.328	**0.027**	**0.008**	**0.008**	**0.010**	**0.021**	**0.002**
Femur length	**rho**	0.020	-0.001	-0.051	-0.099	-0.075	**0.248**	0.169	**0.454**
	P	0.901	0.991	0.664	0.398	0.524	**0.029**	0.075	**<0.0001**
EFW	**rho**	-0.162	-0.083	-0.197	**-0.276**	**-0.247**	**0.347**	**0.285**	**0.415**
	P	0.300	0.477	0.090	**0.017**	**0.033**	**0.002**	**0.012**	**0.0002**
AFI	**rho**	0.091	-0.013	-0.145	-0.138	-0.148	0.209	0.148	-0.018
	P	0.560	0.909	0.218	0.242	0.208	0.069	0.052	0.877
MCA PI	**rho**	-0.066	-0.078	-0.181	**-0.228**	**-0.248**	0.012	0.084	-0.191
	P	0.682	0.517	0.127	**0.014**	**0.035**	0.915	0.648	0.099
UA PI	**rho**	-0.001	-0.115	-0.036	-0.096	-0.054	0.037	-0.015	-0.156
	P	0.999	0.351	0.774	0.435	0.604	0.762	0.667	0.194

Spearman's rho (top value), *P* value (bottom value). Negative values indicate a negative association. MCA = middle cerebral artery pulsatility index, UA = umbilical artery pulsatility index. Values in bold are statistically significant.

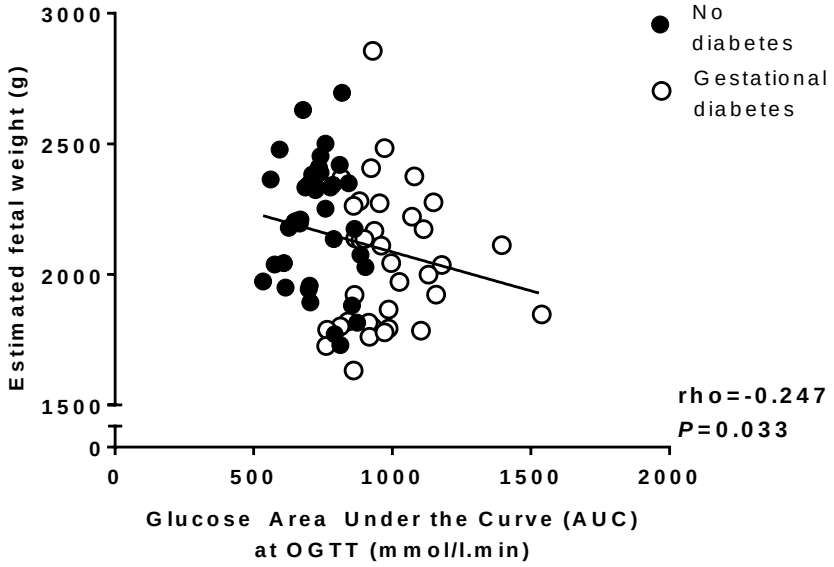

Figure 5.15 Association between maternal glucose AUC at OGTT and EFW at 32-34 weeks gestation
Solid line denotes line of best fit for combined data.

5.5.3 Fetal kidney parameters at 32-34 weeks gestation

5.5.3.1 Normal fetal kidney volume in gestational diabetes

Fetal kidney measurements at 32-34 weeks gestation are presented in **Table 5.9**. There was no difference in left or right kidney length or volume between fetuses of women with and without gestational diabetes. However, the maximum renal pelvis diameters in both left and right kidneys were greater in fetuses of women with gestational diabetes (left: *P*=0.010; right: *P*=0.0002).

There was no difference in any measurement of kidney size or kidney volume between left and right kidneys (length *P*=0.998; width *P*=0.560; depth *P*=0.623, pelvis diameter *P*=0.983; volume *P*=0.397).

Table 5.9 Fetal kidney parameters at 32-34 weeks gestation

		No diabetes (*n*=42)	**Gestational diabetes** (*n*=36)	***P***
Right kidney	**Length (longitudinal), cm**	3.94 ± 0.38	3.98 ± 0.58	0.705
	Width (transverse diameter), cm	2.25 ± 0.36	2.24 ± 0.33	0.615
	Depth (antero-posterior diameter), cm	2.20 ± 0.24	2.23 ± 0.41	0.879
	Volume, cm^3	10.32 ± 2.73	10.66 ± 3.87	0.656
	Maximum renal pelvis diameter, cm	0.21 ± 0.09	0.29 ± 0.12	**0.010**
Left kidney	**Length (longitudinal), cm**	3.89 ± 0.42	4.02 ± 0.49	0.202
	Width (transverse diameter), cm	2.23 ± 0.39	2.19 ± 0.38	0.445
	Depth (antero-posterior diameter), cm	2.16 ± 0.28	2.22 ± 0.38	0.669
	Volume, cm^3	10.07 ± 3.36	10.45 ± 3.97	0.945
	Maximum renal pelvis diameter, cm	0.19 ± 0.08	0.30 ± 0.12	**0.0002**

Data analysed by Wilcoxon rank sum test. Values are mean ± SD.

Mean kidney volume (no diabetes 10.20 ± 2.88 cm^3, gestational diabetes 10.54 ± 3.65 cm^3; *P*=0.644; **Figure 5.16A**) and mean kidney volume relative to EFW (no diabetes 4.61 ± 1.30 cm^3/kg, gestational diabetes 4.99 ± 1.35 cm^3/kg; *P*=0.216; **Figure 5.16B**) were similar in fetuses of women with and without gestational diabetes. There was also no difference in combined kidney volume (no diabetes 20.39 ± 5.76 cm^3, gestational diabetes 20.36 ± 6.07 cm^3; *P*=0.978) or combined kidney volume relative to EFW between groups (no diabetes 9.23 ± 2.54 cm^3/kg, gestational diabetes 9.86 ± 2.65 cm^3/kg; *P*=0.299). Mean fetal kidney volume and combined fetal kidney volume were also analysed with respect to abdominal circumference. There was no difference in either parameter between fetuses of women with and without gestational diabetes (*P*=0.976 and *P*=0.625, respectively).

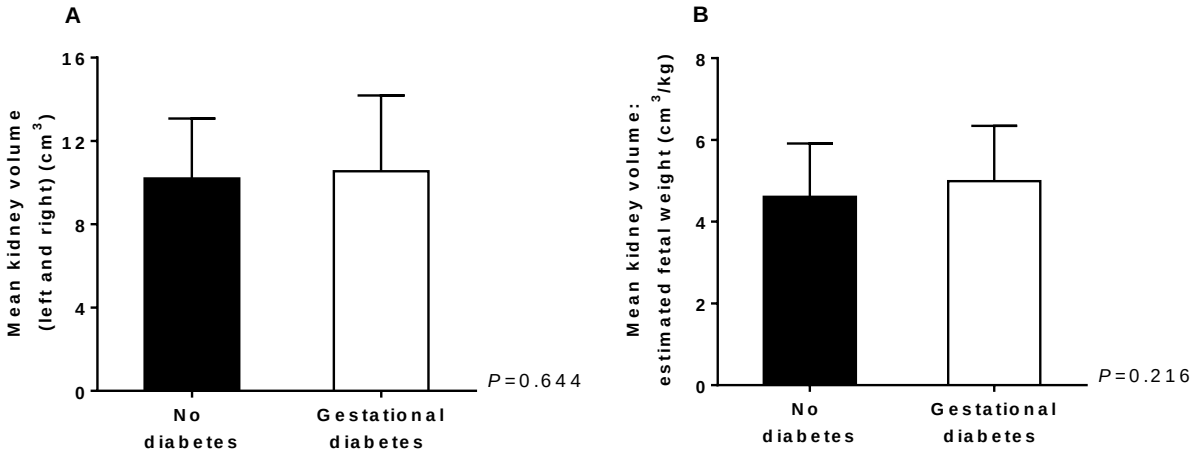

Figure 5.16 Mean kidney volume at 32-34 weeks gestation
A Mean fetal kidney volume and **B** mean kidney volume relative to EFW in women without diabetes and women with gestational diabetes. Data analysed by Wilcoxon rank sum test. Values are mean ± SD. *n*(no diabetes)=42, *n*(gestational diabetes)=36.

Mean fetal kidney dimensions (length, width and depth) were analysed in absolute terms and relative to EFW (see **Table 5.10**). These analyses revealed no difference in absolute kidney lengths or diameters between women with and without gestational diabetes. However, fetuses of women with gestational diabetes exhibited significantly greater mean kidney length relative to EFW (*P*=0.003) as well as greater mean kidney depth relative to EFW (*P*=0.025). Kidney dimensions and kidney volumes were similar in male and female fetuses (data not presented).

Table 5.10 Additional fetal kidney parameters at 32-34 weeks gestation

	No diabetes (*n*=41)	**Gestational diabetes** (*n*=33)	***P***
Mean length, cm	3.91 ± 0.38	4.01 ± 0.46	0.114
Mean width, cm	2.24 ± 0.34	2.22 ± 0.32	0.691
Mean depth, cm	2.18 ± 0.22	2.23 ± 0.37	0.679
Mean length: EFW, cm/kg	1.79 ± 0.24	1.98 ± 0.29	**0.003**
Mean width: EFW, cm/kg	1.02 ± 0.17	1.08 ± 0.18	0.104
Mean depth: EFW, cm/kg	0.99 ± .012	1.09 ± 0.20	**0.025**

Data analysed by Wilcoxon rank sum test. Values are mean ± SD.

Fetal kidney parameters were also assessed with regard to maternal treatment. There was a trend for mean kidney length relative to EFW to be greater in insulin-treated gestational diabetic women than in women treated with diet and exercise (*P*=0.083; **Figure 5.17A**). A similar trend was found for relative kidney depth (**Figure 5.17B**). As mentioned, there was no difference in mean kidney volume relative to EFW when all women with gestational diabetes were compared with women without diabetes (**Figure 5.16B**). However, relative kidney volume was greater in women treated with insulin compared

with women treated with diet and exercise (*P*=0.027) and women without diabetes (*P*=0.025) (**Figure 5.17C**). There was no effect of maternal insulin treatment on other parameters of fetal kidney size (data not shown).

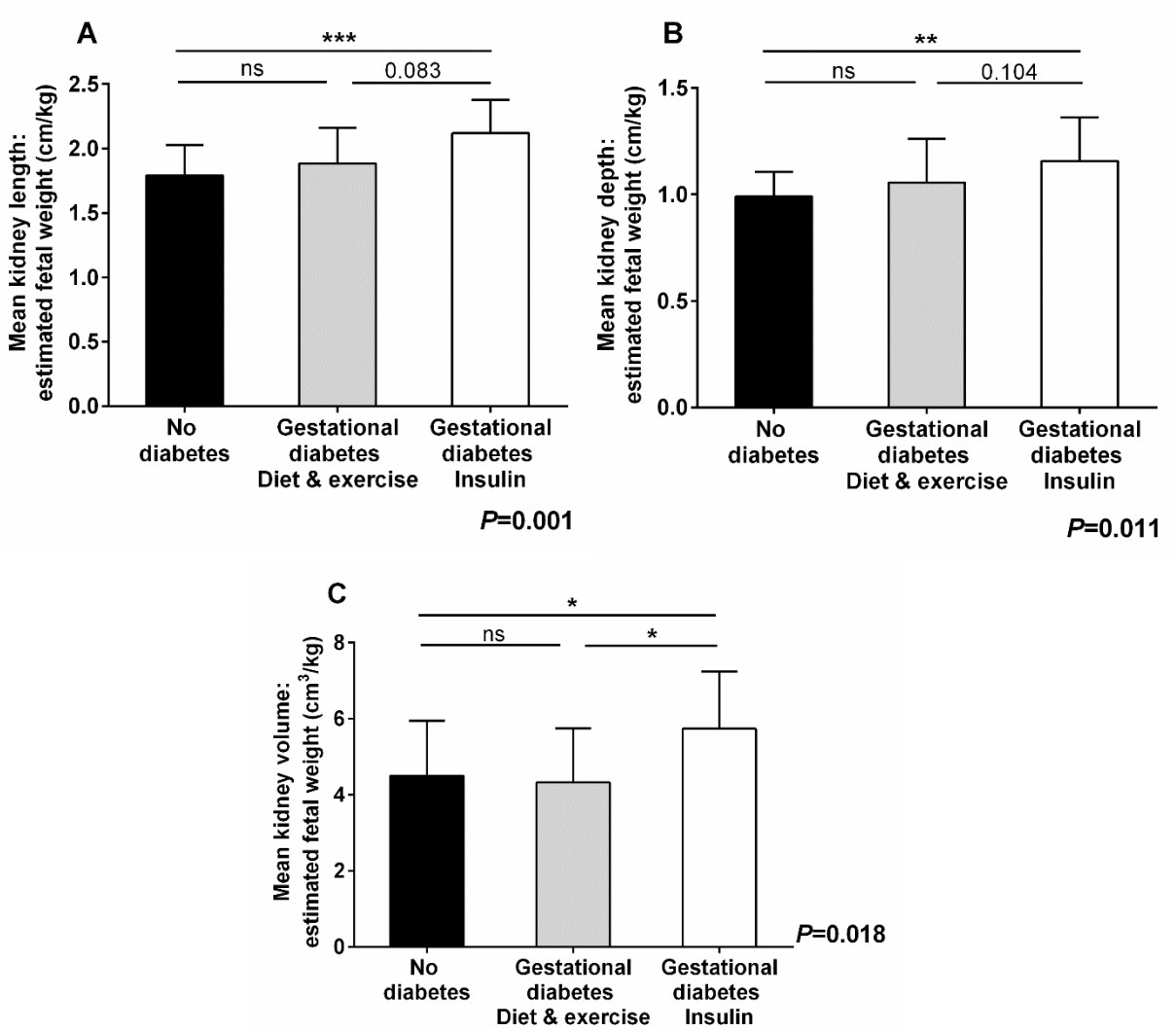

Figure 5.17 Relative kidney length at 32-34 weeks gestation in women treated with and without insulin

A Mean fetal kidney length relative to EFW, **B** mean fetal kidney depth relative to EFW and **C** mean fetal kidney volume relative to EFW at 32-34 weeks gestation in women without diabetes, women with gestational diabetes treated with diet and exercise, and women with gestational diabetes treated with insulin. Analysis by Kruskal Wallis one-way ANOVA and Dunn's multiple comparisons test. Values are mean ± SD. *n*(no diabetes)=42, *n*(gestational diabetes diet and exercise)=23, *n*(gestational diabetes insulin)=12. **P*<0.05, ***P*<0.01, ****P*<0.001.

5.5.3.2 Associations between maternal characteristics and fetal kidney size

Spearman's correlation analyses were used to assess associations between maternal parameters and fetal kidney size (**Table 5.11**). Mean kidney length relative to EFW was positively correlated with 1 hour

and 2 hour glucose values during OGTT and glucose AUC. However, there were no associations between other parameters of fetal kidney size and maternal glucose. Mean kidney length, mean kidney width, mean kidney volume and combined kidney volume were all positively correlated with maternal weight and BMI at booking appointment. EFW and abdominal circumference were positively correlated with fetal kidney volume.

AFI was significantly associated with mean kidney length relative to EFW (rho=0.309, *P*=0.006) but no other kidney parameters.

Table 5.11 Spearman's rank correlation coefficients for associations between maternal and fetal characteristics and fetal kidney size

		Fasting glucose	1 hour glucose	2 hour glucose	Glucose AUC	Weight at booking	BMI at booking	Age at conception	EFW	Abdominal circ.
Mean kidney length	**rho**	0.133	0.121	0.128	0.156	**0.241**	0.208	0.064	0.206	0.207
	P	0.255	0.301	0.272	0.182	**0.034**	0.068	0.576	0.070	0.069
Mean kidney width	**rho**	0.029	0.099	-0.124	0.031	**0.367**	**0.303**	0.080	0.201	0.153
	P	0.809	0.412	0.301	0.797	**0.001**	**0.009**	0.498	0.086	0.193
Mean kidney depth	**rho**	0.039	-0.039	-0.012	-0.026	0.188	0.151	0.141	0.229	0.210
	P	0.746	0.743	0.916	0.829	0.109	0.199	0.229	0.050	0.077
Mean kidney length: EFW	**rho**	0.140	**0.310**	**0.246**	**0.327**	-0.178	-0.151	0.001	**-0.707**	**-0.648**
	P	0.232	**0.007**	**0.033**	**0.004**	0.119	0.187	0.990	**<0.0001**	**<0.0001**
Mean kidney width: EFW	**rho**	0.035	0.148	0.033	0.134	-0.043	-0.063	0.072	**-0.533**	**-0.546**
	P	0.772	0.217	0.787	0.266	0.713	0.590	0.542	**<0.0001**	**<0.0001**
Mean kidney depth: EFW	**rho**	0.033	0.123	0.116	0.143	-0.0214	-0.212	0.079	**-0.642**	**-0.595**
	P	0.782	0.307	0.335	0.235	0.067	0.070	0.503	**<0.0001**	**<0.0001**
Mean kidney volume	**rho**	0.042	0.055	-0.063	0.026	**0.349**	**0.287**	0.155	**0.284**	**0.250**
	P	0.723	0.644	0.594	0.725	**0.002**	**0.011**	0.179	**0.012**	**0.028**
Mean kidney volume: EFW	**rho**	0.053	0.153	0.006	0.124	0.194	0.147	0.105	-0.130	-0.140
	P	0.653	0.195	0.963	0.296	0.094	0.205	0.367	0.263	0.229
Combined kidney volume	**rho**	0.045	0.082	-0.062	0.043	**0.350**	**0.306**	0.157	**0.305**	**0.285**
	P	0.709	0.496	0.609	0.723	**0.002**	**0.008**	0.181	**0.008**	**0.014**
Comb. kidney volume: EFW	**rho**	0.060	0.170	0.006	0.136	0.163	0.120	0.099	-0.114	-0.118
	P	0.620	0.155	0.998	0.260	0.164	0.310	0.400	0.335	0.320

Spearman's rho (top value), *P* value (bottom value). Negative values indicate a negative association. Values in bold are statistically significant.

5.5.4 Birth outcomes

Birth outcomes are presented in **Table 5.12**. The mode of delivery was similar across the two groups although there was a trend for women with gestational diabetes to deliver earlier than women without diabetes (*P*=0.068). There was no difference in arterial and venous umbilical lactate levels or 1 and 5 minute Apgar scores between babies of women without diabetes and babies of women with gestational diabetes. There was no difference in the proportion of SGA or LGA babies. Twice as many babies of women with gestational diabetes were admitted to special care nursery than babies born to women without diabetes, yet this was not of statistical significance. A greater proportion of babies born to gestational diabetic women exhibited hypoglycaemia, jaundice and respiratory distress syndrome, however statistical analysis was not performed due to insufficient observations. The incidence of suspected sepsis was similar in the two groups.

Table 5.12 Birth outcomes

		No diabetes (*n*=39, %)	**Gestational diabetes** (*n*=34, %)	***P***
Mode of delivery, %	Vaginal	15 (38%)	15 (44%)	0.738
	Instrumental	14 (36%)	9 (26%)	0.616
	Elective LUSCS	4 (10%)	5 (15%)	0.823
	Emergency LUSCS	6 (15%)	5 (15%)	0.999
Umbilical lactate, mmol/l	Arterial	5.2 ± 2.9	5.5 ± 3.3	0.936
	Venous	3.8 ± 2.5	3.9 ± 2.2	0.798
Apgar score	1 minute	8.4 ± 1.6	8.2 ± 1.4	0.211
	5 minutes	8.8 ± 0.9	8.8 ± 0.6	0.895
Gestation at birth, weeks		39.6 ± 1.2	39.1 ± 1.2	0.068
Macrosomia, birth weight ≥ 4500g		1 (3%)	0	N/A
LGA: > 90th percentile, %		5 (13%)	3 (9%)	0.864
SGA: < 10th percentile, %		4 (10%)	4 (12%)	0.928
Admission to special care nursery, %		8 (21%)	13 (39%)	0.391
Perinatal outcomes	Hypoglycaemia	1 (3%)	5 (15%)	N/A
	Jaundice requiring phototherapy	1 (3%)	3 (9%)	N/A
	Suspected sepsis	9 (24%)	5 (15%)	0.691
	Respiratory distress syndrome	1 (3%)	4 (12%)	N/A

Data concerning umbilical lactate, Apgar score, and gestational age at birth presented as mean ± SD and analysed by Wilcoxon rank sum test. All other data analysed by population proportions test. N/A indicates insufficient observations to perform statistical analysis. LUSCS = lower uterine segment Caesarean section.

5.5.4.1 Lower birth weight in gestational diabetes

In line with lower EFW, birth weight was 8% lower in babies born to women with gestational diabetes compared with babies of women without diabetes (no diabetes 3476.3 ± 512.4 g, gestational diabetes 3192.9 ± 414.9 g; *P*=0.011; **Figure 5.18**). Head circumference was also lower in babies of women with gestational diabetes (no diabetes 34.5 ± 1.6 cm, gestational diabetes 33.7 ± 1.3 cm; *P*=0.016; **Figure 5.19**) and there was a trend for body length to be reduced (no diabetes 50.2 ± 2.1 cm, gestational diabetes 49.3 ± 2.1 cm; *P*=0.058). Birth weight to length ratio was smaller in babies born to women with gestational diabetes, indicating that babies were lighter for a given length (no diabetes 68.8 ± 7.8 g/cm, gestational diabetes 64.9 ± 7.0 g/cm; *P*=0.026). Ponderal index was however similar between babies of women with and without gestational diabetes (no diabetes 2.70 ± 0.21 g/cm^3, gestational diabetes 2.67 ± 0.33 g/cm^3; *P*=0.680). Birth weight percentile was significantly lower in babies of women with gestational diabetes (*P*=0.010; **Figure 5.20**).

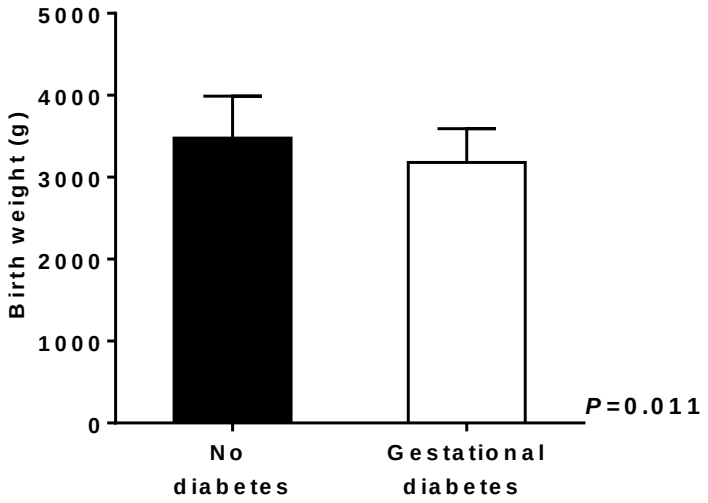

Figure 5.18 Birth weight

Birth weight for babies of women without diabetes and babies of women with gestational diabetes. Data analysed by Wilcoxon rank sum test. Values are mean ± SD. *n*(no diabetes)=39, *n*(gestational diabetes)=34.

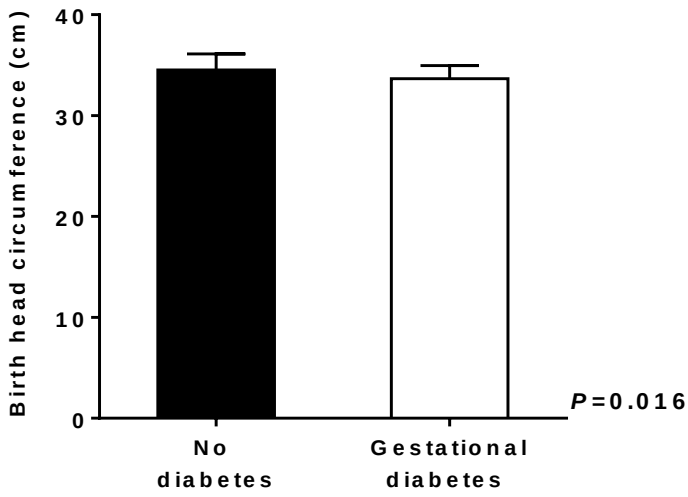

Figure 5.19 Head circumference at birth

Head circumference at birth for babies of women without diabetes and babies of women with gestational diabetes. Data analysed by Wilcoxon rank sum test. Values are mean ± SD. *n*(no diabetes)=39, *n*(gestational diabetes)=34.

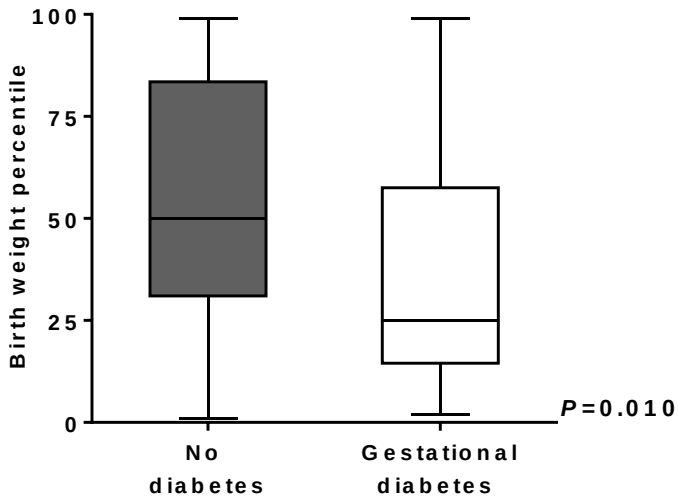

Figure 5.20 Birth weight percentile

Birth weight percentile for babies of women without diabetes and women with gestational diabetes. Data analysed by Wilcoxon rank sum test. Data presented as box and whisker plot. *n*(no diabetes)=39, *n*(gestational diabetes)=34.

There was no difference in birth weight between babies born to women with gestational diabetes who were treated with diet and exercise and women who were treated with insulin (diet and exercise 3120.2 ± 417.3 g, insulin 3208.3 ± 421.0 g; *P*=0.998; **Figure 5.21**). There was also no difference in birth head circumference for babies born to women with gestational diabetes treated by diet and exercise, or insulin (diet and exercise 33.6 ± 1.4 cm, insulin 33.8 ± 1.1 cm; *P*=0.998).

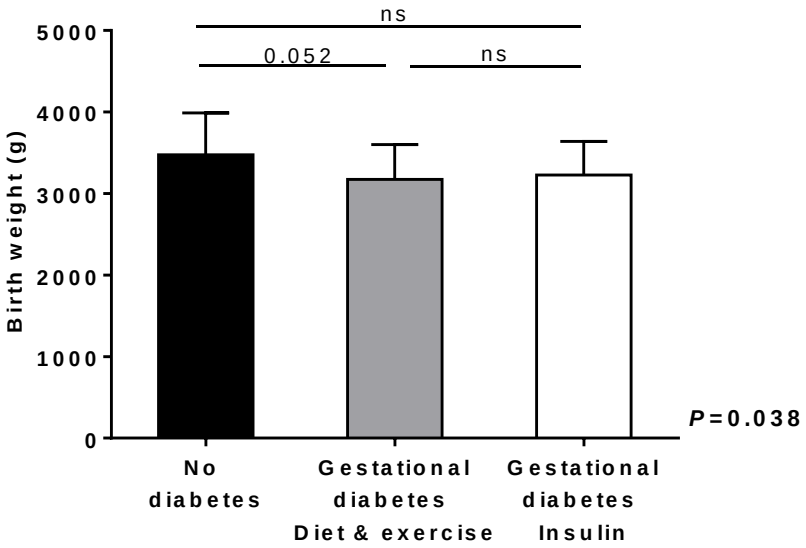

Figure 5.21 Birth weight of babies born to women treated with and without insulin
Birth weight of babies born to women without diabetes, women with gestational diabetes treated with modifications to diet and exercise, and women with gestational diabetes treated with insulin. Analysis by Kruskal Wallis one-way ANOVA and Dunn's multiple comparisons test. Values are mean ± SD. *n*(no diabetes)=39, *n*(gestational diabetes diet and exercise)=23, *n*(gestational diabetes insulin)=12.

There was no statistical difference in the ratio of male to female babies between the two groups (*P*=0.155) despite a higher percentage of boys born to women without gestational diabetes than with gestational diabetes (64% versus 45%). Overall, males were heavier than females at birth (male birth weight 3457.1 ± 504.4 g, female birth weight 3185.0 ± 428.6 g; *P*=0.045), yet when assessed by maternal diabetes status there was no statistical difference in birth weight between males and females in women without diabetes or women with gestational diabetes (**Table 5.13**). The difference in birth weight between babies of women with and without gestational diabetes was more pronounced in males than females, although this was also not of statistical significance (**Table 5.13)**.

Table 5.13 Male and female birth weights

	No diabetes (*n*)	**Gestational diabetes** (*n*)	***P***
Male birth weight (g)	3600.3 ± 501.5 (25)	3233.4 ± 434.1 (16)	0.174
Female birth weight (g)	3254.3 ± 469.4 (14)	3131.1 ± 399.4 (18)	0.999
***P* value**	0.365	0.998	

Data analysed by Kruskal Wallis one-way ANOVA and Dunn's multiple comparison test. Values are mean ± SD.

5.5.5 Altered fetal growth trajectory in gestational diabetes

EFWs and birth weight were plotted to assess fetal growth trajectories across gestation. Analysis by repeated measures ANOVA revealed a significant difference in growth trajectory between fetuses of women without diabetes and women with gestational diabetes (*P*=0.001; **Figure 5.22A**). Weight percentiles were also plotted across gestation to further highlight the differences in growth trajectories between the two groups (*P*=0.014; **Figure 5.22B**). **Figure 5.22B** indicates a slight increase in fetal weight centile between 19-22 weeks and 32-34 weeks gestation in women without diabetes, followed by a decrease before birth. Conversely, in women with gestational diabetes a marked downward centile crossing is observed between 19-22 weeks and 32-34 weeks gestation, followed by a slight decrease again before birth. There was no statistical difference in weight percentiles across gestation in non-diabetic women (*P*=0.244), whereas a significant difference was found for women with gestational diabetes (*P*=0.001). It should be noted that it is unknown when in gestation, between 19-22 weeks and 32-34 weeks gestation, this reduction in fetal weight centiles occurred in the gestational diabetic women.

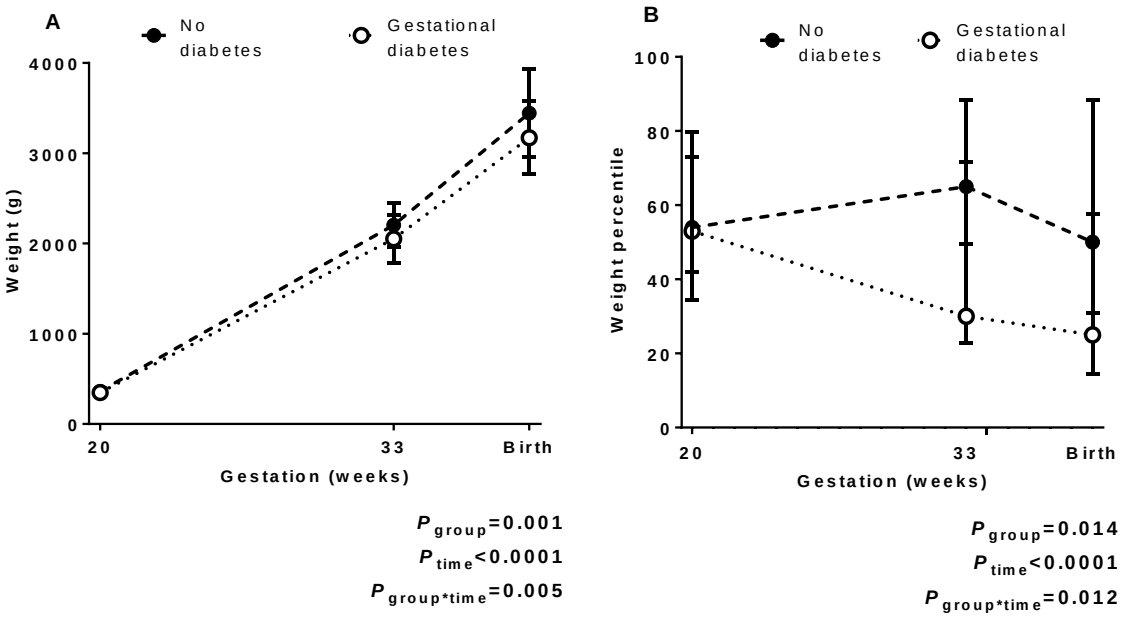

Figure 5.22 Fetal weight trajectory across gestation

A Fetal weight across gestation in women without diabetes and women with gestational diabetes. Data analysed by repeated measures ANOVA. Values are mean ± SD. **B** Fetal weight percentile across gestation in women without diabetes and women with gestational diabetes. Data analysed by repeated measures ANOVA. Values are median ± IQR. *n*(no diabetes)=37, *n*(gestational diabetes)=34.

5.6 Discussion

In the present chapter, women with gestational diabetes were studied to investigate the effects of maternal glucose intolerance on fetal kidney size and fetal growth. Gestational diabetes is typically associated with fetal and neonatal macrosomia. Unexpectedly, lower EFW at 32-34 weeks and lower birth weight were observed in the cohort of women with gestational diabetes. Reduced fetal middle cerebral artery pulsatility index and AFI were also observed in women with gestational diabetes, although these values were within the normal clinical range. Despite these outcomes, the preliminary data presented in this chapter indicates that fetal kidney volume is normal in gestational diabetes. These results and their implications are discussed below.

5.6.1 Maternal characteristics

Several factors place pregnant women at high risk of developing gestational diabetes. These include previous gestational diabetes, advancing maternal age, obesity and ethnic background (Savvidou et al., 2010, Nanda et al., 2011, Teede et al., 2011, ADIPS, 2014). In the present study, there was no difference in maternal age, weight at booking visit, BMI at booking visit or prevalence of previous gestational diabetes between women with and without gestational diabetes. Previous studies have identified a linear trend between the prevalence of gestational diabetes and maternal age and BMI (Callaway et al., 2006, Chu et al., 2007, Templeton and Pieris-Caldwell, 2008, Torloni et al., 2009, Carolan et al., 2012). In the present study, maternal BMI was associated with increasing fasting glucose at OGTT. However, there was no correlation between any other OGTT value (1 hour, 2 hour or glucose AUC) with maternal weight, BMI or age. Given that maternal age, BMI and previous gestational diabetes are well-recognised independent risk factors for gestational diabetes (Nankervis and Conn, 2013) it is surprising that no difference in either variable was observed. This may be related to the moderately small sample size of the present study. It may also reflect that the cohort of women with gestational diabetes were of relatively low-risk, as a number of women diagnosed with gestational diabetes do not have demonstrable risk factors identified at booking appointment (Weeks et al., 1994, Griffin et al., 2000, Teede et al., 2011, Duran et al., 2014). This reiterates the importance of universal testing rather than selective screening based on identified risk factors (Moses and Cheung, 2009).

However, the suggestion that the women in the present study with gestational diabetes were a low-risk population is not supported by the increase in early pregnancy HbA1c. Due to a paucity of studies, HbA1c is currently of limited use for the diagnosis, management and postpartum assessment of women with gestational diabetes. However, recent studies show that elevated early pregnancy HbA1c is predictive of gestational diabetes and overt postpartum diabetes (Katon et al., 2011, Fong et al., 2014, Granada et al., 2014, Hughes et al., 2014). High HbA1c in early pregnancy is also associated with

a need for medication in the treatment of gestational diabetes (Clayton et al., 2012, Alunni et al., 2015), and an increased risk of adverse pregnancy outcomes including congenital anomalies, pre-eclampsia, shoulder dystocia, perinatal death and LGA birth weight (Hughes et al., 2014), although not all studies report such associations (Fong et al., 2014). In the present study, HbA1c measured at first booking visit was elevated in women with gestational diabetes. While there were no associations between HbA1c and fetal outcomes, strong positive correlations were found between HbA1c values in early pregnancy and OGTT values at 24-28 weeks gestation. Early pregnancy HbA1c values were missing in the majority of women with gestational diabetes. HbA1c values were only mildly elevated and no HbA1c value was indicative of overt diabetes (≥ 48 mmol/mol). Nonetheless, the significant increase in early pregnancy HbA1c may indicate that a subset of women with gestational diabetes had underlying, sub-clinical hyperglycaemia in early pregnancy, or even before conception. Indeed, impaired glucose tolerance or pre-diabetes was confirmed in 21% of women in the early post-partum period, however it is difficult to correlate this with early pregnancy HbA1c due to the small sample size.

New recommendations for the testing and diagnosis of gestational diabetes were formulated by IADPSG in 2010. ADIPS have since endorsed these recommendations yet some pathology providers, including Monash Health, continue to use the previous guidelines. Based on Monash Health criteria, 17% of women diagnosed with gestational diabetes exhibited fasting hyperglycaemia (≥ 5.5 mmol/l). If the new ADIPS criteria regarding fasting glucose levels had been applied in the present study, 36% of the cohort would have displayed fasting hyperglycaemia (≥ 5.1 mmol/l). Interestingly, the mean fasting glucose value for women with gestational diabetes was less than 5.1 mmol/l, further signifying that only a few women with gestational diabetes had fasting hyperglycaemia. The majority of women in our cohort exhibited abnormal glucose tolerance, with 89% demonstrating elevated glucose at 2 hours (≥ 8.0mmol/l). If the new ADIPS criteria were applied, only 61% of women would have had elevated glucose at 2 hours (≥ 8.5 mmol/l). Moreover, seven women would no longer be diagnosed with gestational diabetes, highlighting that our cohort included a number of women with mild or borderline gestational diabetes. Note that no women in the control group had glucose levels that would have led to a diagnosis of gestational diabetes under the new ADIPS criteria.

Differences in the magnitude of glucose intolerance and the prevalence of fasting hyperglycaemia raise the question of which is more detrimental to the fetus. Correlation analyses revealed that fetal abdominal circumference, EFW and middle cerebral artery pulsatility index were significantly associated with 2 hour OGTT values, but not fasting glucose. This may not necessarily reflect a lack of association between fasting hyperglycaemia and adverse fetal outcomes, but may be due to the low number of women who presented with fasting hyperglycaemia in our cohort. The findings of the HAPO study revealed increasing fasting, 1 hour and 2 hour plasma glucose levels were similarly predictive of

LGA birth weight, neonatal adiposity, neonatal hyperinsulinaemia and Caesarean delivery with no single measure superior in predicting outcomes (HAPO Study Cooperative Research Group, 2008, HAPO Study Cooperative Research Group, 2009). It has therefore been suggested that any flux in maternal glucose, whether it be in the fasting or postprandial state, can affect the fetus.

5.6.2 Maternal ethnicity

As mentioned, ethnicity is a high-risk factor for gestational diabetes. The latest report from the AIHW found women born overseas have twice the incidence rate of gestational diabetes as women born in Australia (Templeton and Pieris-Caldwell, 2008). When analysed by region, women of South Asian ethnicity have the greatest incidence of gestational diabetes, with odds of developing gestational diabetes up to 4-fold higher than women born in Australia (Anna et al., 2008, Templeton and Pieris-Caldwell, 2008, Drysdale et al., 2012). Other high-risk regions include South East/East Asia, Middle East, Africa and the Pacific Islands (Anna et al., 2008, Templeton and Pieris-Caldwell, 2008, Carolan et al., 2012, Drysdale et al., 2012). Similarly, studies from the U.S. report a greater risk of gestational diabetes in Asian (any), Pacific Islander, Hispanic and black women compared to non-Hispanic white women (Thorpe et al., 2005, Lawrence et al., 2008, Hunsberger et al., 2010). The increased risk of gestational diabetes seen in certain ethnic regions may be related to differences in visceral adipose tissue, body fat percentage, BMI, acculturation, dietary intake and lifestyle behaviours (Chu et al., 2009, Hunsberger et al., 2010).

Clinical profiles have been found to vary between women with gestational diabetes of different racial backgrounds. Compared to Caucasian women, women born in South-East/East Asia have a lower BMI, lower fasting glucose level and reduced need to start insulin therapy, yet a higher 2 hour glucose level during an OGTT (Gunton et al., 2001, Templeton and Pieris-Caldwell, 2008, Hedderson et al., 2012, Wong, 2012). South Asian women also have a lower BMI than Caucasian women, yet higher 2 hour glucose levels during an OGTT and a stronger family history of diabetes (Wong, 2012). Despite these differences, insulin resistance and pancreatic β cell function are reported to be comparable between Caucasian, South Asian and South-East/East Asian women with gestational diabetes (Shelley-Jones et al., 1993, Gunton et al., 2001). When looking at pregnancy outcomes, women with gestational diabetes of South Asian and South-East/East Asian descent have lower birth weights, fewer LGA infants and more SGA infants than Caucasian women with gestational diabetes (Wong, 2012). The ethnic differences presented in this study may contribute to the similar maternal BMI and lower infant birth weights observed in women with gestational diabetes.

Recently, the INTERGROWTH-21st project produced international standards for newborn anthropometrics. In this multi-country project, birth weight, length and head circumference were

found to be essentially identical across eight urban populations where the health and nutritional needs of the mother were met, adequate antenatal care was provided and where there were no major environmental constraints on growth (Villar et al., 2014). The INTERGROWTH-21st project showed that neonatal anthropometric parameters are similar across multiethnic populations at low risk of altered fetal growth, reducing any potentially confounding effect of ethnic variation in study populations. However, it is unlikely that these same growth charts can be applied across different ethnicities in the presence of obstetric complications such as gestational diabetes.

5.6.2.1 Assessing the role of maternal ethnicity in the present study

Recent birth statistics from the AIHW report that 29% of women who gave birth in Australia in 2011 were themselves born outside of Australia (Hilder et al., 2014). Monash Health provides services to an ethnically diverse antenatal population, where up to 60% of births are in non-Australian born women (Teh et al., 2011). The present study recognises that there are ethnic differences between women with gestational diabetes and women without diabetes in pregnancy. These differences are due, in part, to the high prevalence of women born in South Asia diagnosed with gestational diabetes, and the relatively low prevalence of women of Caucasian ethnicity diagnosed with gestational diabetes.

Unfortunately, this study is underpowered to assess the role of maternal ethnicity. Considering the primary outcome of fetal kidney size, we aimed to detect a 15% difference between women with and without gestational diabetes. In the non-diabetic population, the standard deviation of mean kidney volume was 28%. Based on this and a statistical power of 0.8 (α=0.05), 28 subjects per ethnic group and diabetes diagnosis are required to assure the analysis is adequately powered. As it currently stands, the number of Caucasian, South Asian and South East/East Asian women with or without gestational diabetes is well below this number, especially concerning South Asian women without diabetes and Caucasian women with gestational diabetes.

The data presented in this thesis is preliminary and obtained as part of an ongoing study. The governing ethics committees have approved 85 women to be recruited per group, and recruitment will continue throughout 2015 and 2016. Sub-analyses to assess what role maternal ethnicity has on maternal parameters, maternal glucose profiles in pregnancy and fetal growth, and to assess, in turn, what impact these variables have on fetal kidney size, will be performed once sufficient women have been recruited. In the interim it is important to recognise that differences in maternal and fetal outcomes reported in the present study may be influenced or biased by differences in maternal ethnicity between groups, as eluded to above.

5.6.3 Fetal size and growth

Gestational diabetes is associated with altered fetal growth patterns. Untreated gestational diabetes and impaired glucose tolerance are typically associated with a greater incidence of LGA birth weight and macrosomia compared to non-diabetic women (Östlund et al., 2003, HAPO Study Cooperative Research Group, 2008, Catalano et al., 2012, Black et al., 2013, Graves et al., 2015). The increase in birth weight seen in gestational diabetes is attributed to increased fetal fat mass and accelerated abdominal growth, particularly of the fetal liver and abdominal wall subcutaneous fat, early in the third trimester (Kehl et al., 1996, Schaefer-Graf et al., 2011, Hammoud et al., 2013). Treating gestational diabetes with dietary advice, blood glucose monitoring and insulin therapy reduces the risk of neonatal macrosomia and LGA infants (Langer et al., 1989a, Crowther et al., 2005, Langer et al., 2005, Landon et al., 2009, Nanda et al., 2011, Graves et al., 2015). However, some studies continue to report an elevated rate of macrosomia in treated women compared with the normal obstetric population (Shand et al., 2008, Alberico et al., 2014).

Considering that gestational diabetes is typically associated with fetal overgrowth, which may be normalised by glycaemic control, the findings of the present study were unexpected. EFW was lower in women with gestational diabetes at 32-34 weeks gestation, consistent with reduced weight at birth. The decrease in EFW at 32-34 weeks gestation was primarily driven by a reduction in abdominal circumference. Abdominal circumference was unfortunately not measured at birth, however ponderal index, a measure of leanness and proportionate growth, was similar between groups. Conversely, the ratio of birth weight to birth length was lower in infants born to women with gestational diabetes implying asymmetric growth. Interestingly, fetal biometry and EFW were not different between groups at 19-22 weeks before the diagnosis of gestational diabetes. Fetuses of women with gestational diabetes displayed downward centile crossing from mid-gestation, whereas no difference in weight percentiles across gestation was observed in non-diabetic women. The findings of reduced estimated fetal weight and birth weight in the present study should be taken into account when comparing these findings to other studies.

The mean birth weight for babies born to women without diabetes in the present study was 3476g. This is similar to the average live-born birth weight reported in Australia of 3367g (Hilder et al., 2014) and very similar to the mean birth weight at 40 weeks reported at Monash Health. Birth weight was assessed by sex and maternal diabetes status. While not of statistical significance, males born to women without diabetes had the highest birth weight. It is recognised that analysis by sex and maternal diagnosis is not sufficiently powered given the relatively small sample size. It will be interesting to see whether the higher percentage of male births in women without diabetes, who are noticeably heavier, persists in a larger study population.

Very few studies have reported lower birth weight in pregnancies complicated by gestational diabetes. Langer et al. (1989b) found tight glycaemic control in women with gestational diabetes (a mean blood glucose level < 4.7 mmol/l throughout pregnancy) was associated with a high rate of SGA infants. Such aggressive treatment and insulin therapy can reduce fetal growth by compromising fetal nutrient supply and reducing fetal insulin levels (Parikh et al., 2007). When assessed by maternal treatment, the reduction in EFW at 32-34 weeks gestation was more pronounced in women requiring insulin than women treated with modifications to diet and exercise alone. Treatment intervention is typically initiated at 30 weeks gestation at Monash Health, with the majority of women who require insulin starting pharmacotherapy at 30-32 weeks gestation. Given the small time-frame between commencement of insulin therapy and ultrasound at 32-34 weeks, it is unlikely that 'overtreatment' accounts for the reduced EFW at 32-34 weeks gestation. If this was the case it would be reasonable to predict that birth weights would also be lower in women treated with insulin. However, similar reductions in birth weight were observed in women with gestational diabetes regardless of treatment type. The need for insulin therapy indicates that these women had worse glycaemic profiles than women who could maintain glycaemic control with modifications to diet and exercise alone. Women treated with insulin may have had greater perturbations in glucose metabolism, which may have contributed to poorer fetal outcomes at 32-34 weeks gestation. However, it should be noted that these results are based on 12 women treated with insulin and 23 women treated with modifications to diet and exercise. Analysis by maternal treatment type is not sufficiently powered and further analysis in a larger sample size is required.

Previously, Barnes et al. (2013) reported a greater risk of SGA infants in women with low maternal weight gain prior to and after the diagnosis of gestational diabetes. Unfortunately, maternal weight gain was not adequately monitored in the present study. Maternal weight gain has been previously assessed in women with and without gestational diabetes attending Monash Health maternity services (Stewart et al., 2012). They found reduced gestational weight gain in women with gestational diabetes within the first 4 weeks following treatment initiation, implicating that a model of care for gestational diabetes (a diet and lifestyle education session, frequent clinic review and home glucose monitoring) may motivate women to limit gestational weight gain. Incidentally, Stewart et al. (2012) also reported significantly lower birth weights in women with gestational diabetes than women without diabetes, with comparable newborn weights and maternal demographics to that reported in the present study. It therefore seems that the population of women with gestational diabetes attending Monash Health maternity services have smaller babies than women without diabetes.

In the present study it appears that fetal size in women with gestational diabetes is adversely affected at a time in gestation between 19-22 weeks and 32-34 weeks gestation. Precisely when this reduced

rate of fetal growth occurs, and what triggers it, is unknown. Whether the reduction in fetal weight at 32-34 weeks gestation which persists at birth is a consequence of gestational diabetes itself, reduced gestational weight gain shortly after diagnosis, insulin therapy or is related to differences in ethnicities between groups requires further attention.

5.6.4 Fetal wellbeing

In addition to reduced fetal weight, lower AFI and fetal middle cerebral artery pulsatility index were observed in women with gestational diabetes at 32-34 weeks gestation. Diabetes in pregnancy is a recognised risk factor for increased amniotic fluid volume. The main source of amniotic fluid is fetal urination, and thus fetal hyperglycaemia can lead to fetal polyuria and an increase in amniotic fluid (Hamza et al., 2013). Low AFI (<5cm, termed oligohydramnios) is associated with intrauterine growth restriction (IUGR), premature rupture of the membranes and fetal renal anomalies. Abnormal placentation and placental insufficiency also contribute to low AFI (Gilbert and Brace, 1993). Studies evaluating middle cerebral artery pulsatility index in gestational diabetes report either normal or elevated values (Fadda et al., 2001, Leung et al., 2004, Shabani Zanjani et al., 2014). Low middle cerebral pulsatility index is reported in IUGR and SGA, and indicates a reduction in vascular resistance in the fetal cerebrum, re-distribution of cardiac output to the brain, and a 'brain-sparing' effect (Wladimiroff et al., 1986, Mari and Deter, 1992).

While within the normal clinical range, the changes in fetal weight, fetal circulation and amniotic fluid volume seen in the present study may indicate underlying placental dysfunction or sub-clinical placental insufficiency in our cohort of women with gestational diabetes. An additional consideration is the increase in early pregnancy HbA1c observed in these women, which may contribute to mild placental dysfunction. While a statistically significant difference in early pregnancy HbA1c, fetal weight, middle cerebral artery pulsatility index, AFI and birth weight were observed, it is important to question whether these changes are of biological significance as all were within the normal clinical range. Moreover, umbilical artery pulsatility index, a measure of placental vascular resistance, was similar between groups. Further investigation of placental function and fetal/placental haemodynamics in gestational diabetes is warranted yet beyond the scope of the present study.

5.6.5 Fetal kidney size

In the present study, fetuses of women with gestational diabetes were found to have lower EFW at 32-34 weeks gestation as well as reduced AFI and middle cerebral artery pulsatility index which may indicate lower vascular resistance in the fetal brain. Despite these adverse outcomes, fetal kidney size at 32-34 weeks gestation was largely unaffected by gestational diabetes. Fetal kidney dimensions and volume were assessed separately for the left and right kidney, with no difference between left and

right kidney measurements. This is supported by Gloor et al. (1997) and Konje et al. (1997) who found no significant difference in measurements from left and right fetal kidneys, although a large study of 1215 fetuses reported larger right kidney width, depth and volume at 30 weeks gestation (Verburg et al., 2007).

Maximum renal pelvis diameters were greater in fetuses of women with gestational diabetes. Pelvic dilatation or pyelectasis is a relatively common finding detected by obstetric ultrasound that in most cases is physiological and resolves spontaneously, yet in some cases may indicate a urinary tract anomaly (Hothi et al., 2009). Fetal pyelectasis is diagnosed if the renal pelvis diameter measures ≥ 7mm in the third trimester. No renal or urinary tract malformations were found in the present study population and all measurements of fetal renal pelvis diameters were below the diagnostic criteria. Thus the increase in fetal maximum renal pelvis diameter, while interesting, is not likely to be of clinical significance.

Fetuses of women with gestational diabetes had similar left, right and mean kidney dimensions (longitudinal length, transverse diameter and antero-posterior diameter) to fetuses of women without diabetes, despite reduced fetal weight. Mean and combined kidney volumes were also similar in fetuses of women with and without gestational diabetes. When assessed relative to EFW at 32-34 weeks gestation, fetuses of women with gestational diabetes displayed greater mean kidney length and mean kidney depth. This may indicate that fetal kidneys grow normally in the longitudinal and antero-posterior planes and are not affected by gestational diabetes, despite gestational diabetes adversely affecting fetal weight and abdominal circumference. Fetal kidney volume was however not increased in gestational diabetes when adjusted for EFW or abdominal circumference. In contrast to that observed for kidney length and depth, this finding suggests that overall fetal kidney size is proportionate to fetal size. Alternatively, kidney length and depth may be more sensitive markers of kidney growth than kidney volume, considering that kidney volume is estimated from three measurements and assumes an ellipsoid shape.

The increase in kidney length relative to EFW was more pronounced in women treated with insulin than women treated with diet and exercise alone. Relative fetal kidney volume was similar when assessed across all women with gestational diabetes but was significantly increased in the sub-set of women requiring insulin therapy. These outcomes likely reflect the greater reduction in EFW seen in fetuses of women treated with insulin.

Fetal kidney volume was positively associated with maternal weight at booking, maternal BMI and EFW. Mean kidney length correlated with maternal weight at booking, and had a tendency to correlate with maternal BMI and EFW as well. Similar associations between increasing maternal weight, fetal

weight and fetal kidney size have also been reported (Cohen et al., 1991, Gloor et al., 1997, Verburg et al., 2007). When assessing maternal glucose values at OGTT, 1 hour glucose, 2 hour glucose and glucose AUC were found to positively correlate with mean kidney length relative to EFW. This highlights that increases in maternal glucose are associated with increases in fetal kidney length when adjusted for fetal weight. Maternal glucose did not correlate with any other parameter of fetal kidney size.

Previous studies assessing the effect of maternal diabetes on fetal kidney volume are limited. Verburg et al. (2007) performed a comprehensive analysis assessing the effect of various maternal and fetal factors on fetal kidney volume in late gestation in 1215 pregnancies. Maternal diabetes (pre-existing and gestational diabetes) had no association with fetal kidney volume at 30 weeks gestation, although it is important to note only 16 cases of maternal diabetes were reported in the study population. Neves et al. (2013) performed repeat measurements of fetal kidney volume across gestation in 92 women with gestational diabetes considered to have good glycaemic control. Compared to 339 women without diabetes, fetal kidney volume was consistently increased between 22-38 weeks gestation in women with gestational diabetes. The authors found that median fetal kidney volume in gestational diabetes was significantly larger than the 75th percentile kidney volume in the non-diabetic group, with an approximate 9% increase in fetal kidney volume at 32-34 weeks gestation. This study has several limitations however, as almost half of the gestational time points had very few women with gestational diabetes (*n*=2-9), and data concerning fetal weight and birth weight were not presented. This makes it difficult to assess whether gestational diabetes stimulated fetal kidney growth directly or whether kidney volume was increased in proportion to fetal size and overall fetal growth.

Kehl et al. (1996) assessed fetal growth velocities by ultrasound in appropriate for gestational age (AGA) and LGA offspring of diabetic women. While LGA offspring had significantly greater growth velocities for subcutaneous fat, abdominal circumference and liver in the third trimester, the growth velocity of fetal kidney length was similar in AGA and LGA offspring. Likewise, an autopsy study of LGA infants from diabetic mothers found kidney weight was not increased compared to controls despite an increase in other lean tissues including the heart, lungs, liver, spleen, adrenals and pancreas (Naeye, 1965). These studies hypothesised that, unlike fetal adipose tissue and liver, the fetal kidney is not sensitive or responsive to increasing fetal insulin in maternal hyperglycaemia. These findings and those of the present study indicate that fetal kidney size is not targeted in maternal diabetes. This is in contrast to the study by Neves et al. (2013) who suggested that the fetal kidney is subject to organomegaly in gestational diabetes.

Fetal renal perfusion was not measured in the present study, but may be an important consideration. Fetal urine is the main component of amniotic fluid, and AFI correlates well with adequacy of fetal

renal perfusion (Hebbar et al., 2015). Amniotic fluid volume was positively associated with relative fetal renal length in the present study and associates with fetal kidney volume in other studies (Verburg et al., 2007). The slight reduction in AFI in fetuses of women with gestational diabetes may therefore reflect a reduction in fetal urine production and potentially a reduction in renal function. The decrease in middle cerebral artery pulsatility index in gestational diabetes may also adversely affect fetal renal perfusion. A reduction in middle cerebral artery resistance indicates redistribution of fetal blood flow in favour of the brain. If blood was preferentially shunted away from the kidneys to spare the fetal brain and heart, reduced blood flow to the kidneys may disturb development. Further studies that incorporate fetal renal artery Doppler flow velocity to assess blood flow re-distribution in gestational diabetes are warranted.

The accuracy of 2D ultrasound in measuring fetal kidney volume should also be considered. The majority of studies that assess fetal kidney volume assume the kidney is an elongated ellipsoid shape. The classic ellipsoid method is easy to implement yet has been shown to underestimate fetal renal volume (El Behery et al., 2012). Moreover, it has been argued that the kidney is not a true ellipsoid (Yu et al., 2000). 3D ultrasonography offers a more accurate estimate of volumes than 2D techniques (Riccabona et al., 1996). 3D techniques utilise a multiplanar imaging method to generate a series of area traces along the longitudinal axis of the kidney or by rotating the kidney around its axis, to which software automatically calculates renal volume (Hsieh et al., 2000, Yoshizaki et al., 2013). Despite improved accuracy, the application of 3D ultrasonography is limited as it is expensive, time-consuming and not routinely available. Therefore, for practical reasons 2D ultrasound is still widely used to measure fetal kidney volume including in the present study.

5.6.6 Concluding remarks

Gestational diabetes is an increasingly common complication of pregnancy that is typically associated with fetal overgrowth. Unexpectedly, lower fetal weight was observed at 32-34 weeks gestation and persisted at birth in the cohort of women with gestational diabetes attending maternal services at Monash Health. Reduced fetal middle cerebral artery pulsatility index and AFI were also observed, although it should be noted that values were within the normal clinical range. The mechanism for the decline in fetal growth trajectory in gestational diabetes is unclear however a possible explanation is low-level placental dysfunction. Treatment intervention is typically initiated at 30 weeks gestation and is unlikely to explain the difference in EFW at 32-34 weeks gestation. Despite these outcomes, the preliminary data presented in this chapter suggests that fetal kidney volume is normal and is not affected by gestational diabetes. Gestational diabetes was however associated with greater fetal kidney length and depth following adjustment for EFW, which may indicate that fetal kidney growth in the longitudinal and antero-posterior planes is not affected by gestational diabetes, despite

gestational diabetes adversely affecting fetal weight and abdominal circumference. As kidney volume is estimated from three measurements and assumes an ellipsoid shape, fetal kidney length and diameter may be more sensitive markers of fetal kidney growth than kidney volume. It will be interesting to see how fetal kidney volume and kidney length dimensions are affected by gestational diabetes in a larger sample size.

While no difference in fetal kidney volume was observed in the present study, gestational diabetes may alter other parameters of fetal kidney development, including nephron formation, which may affect renal function. Further studies are required to assess fetal renal perfusion as well as renal function in babies born to women with gestational diabetes. Further studies assessing fetal growth and kidney size in women with poorly controlled gestational diabetes are also warranted. However, women with poor glycaemic control are expected to be a minority and may be difficult to capture in a research study. It would instead be interesting to assess fetal growth and fetal kidney size in women displaying more severe forms of gestational diabetes. These women could be selected for based on a higher early pregnancy Hba1c, upper-range fasting or 2 hour glucose levels of the 24-28 week OGTT, or by their requirement for insulin therapy. The present study included a number of women with mild or borderline gestational diabetes. Moreover, preliminary analyses in women requiring insulin treatment revealed more pronounced effects than in women treated with diet and exercise. In a larger study population, excluding mild cases of gestational diabetes and selecting women with greater perturbations in glucose metabolism in pregnancy may strengthen the outcomes reported in this study.

In addition, it is important to note the ethnic differences in this cohort, specifically a low number of Caucasian women with gestational diabetes and a high number of South Asian women with gestational diabetes. The data presented in this thesis is part of an ongoing study and final data analysis will incorporate maternal ethnicity. Final data analysis will also include women diagnosed with gestational diabetes in early pregnancy, as well as women with Type 1 and Type 2 diabetes in pregnancy, to elucidate the effect of all types of maternal diabetes on human kidney development.

CHAPTER SIX

GENERAL DISCUSSION

The obesity epidemic and increases in maternal age have contributed to a growing number of pregnancies complicated by maternal glucose intolerance. Gestational diabetes is the most common complication of pregnancy and is identified in 5-17% of pregnancies in Australia (Yue et al., 1996, Shand et al., 2008, Templeton and Pieris-Caldwell, 2008, AIHW, 2010). This figure is set to rise as new guidelines for the diagnosis of gestational diabetes are implemented (Moses et al., 2011, Sacks et al., 2012). Pre-existing Type 2 diabetes is reported in 2% of pregnancies in Australia, however this is likely an underestimate considering 14% of women of reproductive age exhibit undiagnosed impaired glucose tolerance or pre-diabetes (Dunstan et al., 2002, AIHW, 2008, AIHW, 2010). This may potentially lead to a significant number of women with unrecognised pre-gestational Type 2 diabetes who are later identified at 24-28 weeks gestation by routine testing for gestational diabetes.

The majority of women diagnosed with gestational diabetes have pregnancy-related glucose handling abnormalities, characterised by insulin resistance and hyperglycaemia that develops in the second trimester. While these women typically undergo an OGTT at 24-28 weeks gestation, many may not commence treatment and self-monitor glucose levels until well into the third trimester. The developing fetus may therefore be exposed to a window of glucose intolerance. This window of exposure would be even more pronounced in women with previously undiagnosed Type 2 diabetes. Glucose intolerance in pregnancy has known health implications for the offspring (HAPO Study Cooperative Research Group, 2008, Simeoni and Barker, 2009, Hay, 2012), yet the effect on offspring renal development and subsequent renal function is poorly characterised. The developing kidneys are considered to be particularly susceptible to an adverse intrauterine environment because their development spans almost the entire length of gestation, with nephrogenesis completed shortly before term birth in humans. A window of untreated hyperglycaemia in women with undiagnosed Type 2 diabetes or gestational diabetes may therefore coincide with a proliferative period of nephron formation and may be detrimental to fetal kidney development and subsequent renal health.

In rodents, STZ-induced diabetes is the most common model used to assess the effect of diabetic pregnancy on offspring kidney development. STZ-induced diabetes is a model of Type 1 diabetes that elicits severe and persistent fasting hyperglycemia in the dam as well as profound offspring growth restriction. Experimental studies that utilise STZ report a deficit in offspring nephron number (Amri et al., 1999, Tran et al., 2008, Hokke et al., 2013), yet are confounded by offspring growth restriction. While these studies have contributed to our understanding of how high glucose affects the developing kidney, the translation to human maternal diabetes is limited, given they only model the condition seen in a small subset of pregnancies with poorly controlled Type 1 diabetes also complicated by pre-eclampsia and/or vascular disease. Despite pre-gestational Type 2 diabetes and gestational diabetes

being increasingly common complications of pregnancy, to date, no rodent study has investigated how glucose intolerance affects the developing kidney.

The purpose of this thesis was to assess kidney development in rodent models that better mimic the clinical condition, whereby the maternal phenotype is characterised by increased body weight, mild-moderate hyperglycaemia, insulin resistance and post-prandial hyperglycaemic excursions, without the confounding effects of offspring growth restriction. Previously, high fat feeding in mice and rats has been used to model obesity-associated pre-gestational Type 2 diabetes (Holemans et al., 2004, Samuelsson et al., 2008, Liang et al., 2009a, Nivoit et al., 2009, Li et al., 2013), and leptin receptor deficient mice have been used to model gestational diabetes (Kaufmann et al., 1981, Lawrence et al., 1989, Yamashita et al., 2001, Yamashita et al., 2003, Lambin et al., 2007, Stanley et al., 2011). In the present thesis, high fat fed mice and *Lepr*$^{db/+}$ mice were utilised to explore the effects of glucose intolerance in pregnancy on offspring kidney development.

The first study aimed to: (i) characterise a mouse model of pre-gestational Type 2 diabetes utilising a diet high in fat; (ii) assess if exposure to obesity-associated pre-gestational Type 2 diabetes affects growth and kidney development in offspring during embryogenesis and in early postnatal life; (iii) assess if maternal Type 2 diabetes affects postnatal offspring growth, glucose tolerance and body composition in adulthood; and (iv) assess whether perturbations in kidney development have long-term consequences for renal function and morphology. The second experimental study aimed to: (i) characterise the maternal phenotype of *Lepr*$^{db/+}$ mice as a model of gestational diabetes; and (ii) assess whether gestational diabetes alters early body growth and kidney development in offspring.

6.1 High fat fed dams do not model glucose intolerance in pregnancy

As expected, female mice fed a high fat diet for 6 weeks exhibited increased body weight and glucose intolerance prior to pregnancy. However, this phenotype was not maintained in late gestation. Unexpectedly, glucose profiles in high fat fed dams did not further increase between the pre-gravid state and late gestation. This indicates that high fat fed dams had reached a threshold of glucose intolerance after 6 weeks of high fat feeding that was not exacerbated in pregnancy. Stable glucose profiles in high fat fed dams combined with exacerbated glucose intolerance in normal fat fed dams in the latter half of gestation resulted in similar glucose profiles at E15.5 and E18.5.

A limitation of this study was that GTTs were first performed in pregnant dams at E15.5 and therefore the duration of glucose intolerance in high fat fed dams, and the length of gestation in which embryos were exposed to excess glucose, is unknown. It is possible that there was only limited or no exposure to maternal glucose intolerance during the period of embryonic kidney development. Given

nephrogenesis begins at approximately E12-13, it can be concluded that C57BL6 mice fed a high fat diet are not a suitable model of glucose intolerance in pregnancy for the study of renal development.

6.1.1 Increased nephron endowment in offspring of high fat fed dams

Offspring of dams fed a high fat diet were macrosomic at E18.5, had a tendency to be heavier at PN21 and presented with an elevated nephron endowment that was established relatively early in nephrogenesis.

High fat fed mice displayed variation in susceptibility to glucose intolerance prior to pregnancy. An advantage of defining high fat fed dams with either normal or impaired pre-pregnancy glucose tolerance was the ability to isolate the contribution of maternal high fat feeding *per se* on offspring kidney development. Nephron number was increased in offspring of high fat fed dams regardless of maternal weight and glucose profiles prior to pregnancy. Therefore, we propose the augmented offspring nephron number is likely due to constituents of the high fat diet promoting placental nutrient transfer, fetal growth and nephron formation. These results further confirm the high fat fed mouse as a model of fat feeding rather than maternal glucose intolerance.

Offspring of high fat fed dams displayed a marked increase in nephron number at E18.5 despite exhibiting normal nephron number and ureteric tip number just 3 days prior. This may reflect accelerated ureteric branching morphogenesis after E15.5 or an increase in the number of nephrons formed in each arcade. The length of time required for a nephron to form (from renal vesicle to glomerulus) in the mouse is an interesting question, yet, to our knowledge, has not been previously addressed. Whether extra branching events and the formation of S-shaped bodies or nephron arcades, detectable by PNA histochemistry, can occur within 3 days to induce a 25% increase in nephron number is unknown. The marked increase in nephron formation between E15.5 and E18.5 may also reflect an abundance of immature glomerular structures at E15.5, which do not stain with PNA and were therefore not counted in the present study. Over an additional 3 days, these glomeruli may mature and their podocytes may begin to express galactosamine residues detectable by PNA histochemistry, producing the 25% difference observed at E18.5.

6.1.2 Potential mechanisms of altered nephron endowment in offspring of high fat fed mice

Maternal high fat feeding is one of the few environmental insults found to augment offspring nephrogenesis. It is currently unknown whether a specific nutrient or a range of dietary constituents was responsible for the increased nephron number in the high fat fed offspring. Future studies should aim to identify the underlying mechanisms of this augmented nephrogenesis, particularly the effect of maternal high fat feeding on placental development. The presence of fetal overgrowth and accelerated

nephrogenesis at E18.5 but not E15.5 in offspring of high fat fed dams may be related to differences in placental maturity and/or nutrient transfer between normal fat and high fat fed dams. Placentae were collected at E15.5 and E18.5 and alternatively fixed or frozen, and stored. Real time PCR and Western blot analyses could be utilised to assess gene and protein expression of placental nutrient transporters including GLUTs, FATPs, FATs and amino acid transporters. Immunohistochemistry could be utilised to localise these transporters to the fetal or maternal membranes. Stereology could be employed to quantify the surface area and volume of maternal blood spaces and fetal capillaries, as well as interhemal membrane thickness and capillary lengths (Coan et al., 2004). Moreover, fetal blood, amniotic fluid and maternal blood were collected in the present study and could be used to assess lipid profiles in the maternal and fetal compartments and to further characterise glucose: insulin ratios in the fetal circulation.

A full analysis of the molecular regulation of kidney development in the high fat model was beyond the scope of the present study. However, E18.5 kidneys were collected from a cohort of high fat and normal fat fed dams and frozen and stored. Future studies should assess a possible role for insulin in promoting nephrogenesis in the maternal high fat environment. Insulin has been shown to have a mitogenic effect on the developing kidney, as metanephric explants undergo hypertrophy and display increased nascent nephron formation and hyperplasia in the cortical nephrogenic zone in the presence of insulin (Liu et al., 1997). Moreover, IR and IGF-IR are highly expressed in ureteric epithelium and metanephric mesenchyme in mid gestation (Liu et al., 1993, Liu et al., 1997). Real time PCR, Western blot and immunohistochemistry could be employed to quantify and localise the expression of growth factors in the fetal kidney, with a particular emphasis on the insulin signalling pathway and other pathways known to promote fetal growth that may have a role in nephrogenesis. Gene expression and protein studies should also incorporate markers of ureteric tree development, nephron progenitor cell populations and key regulators of nephrogenesis.

6.1.3 Metabolic health and renal function in adult offspring of high fat fed dams

Exposure to maternal high fat feeding and pre-gestational Type 2 diabetes (albeit limited) had surprisingly little effect on offspring metabolic health. Despite exhibiting macrosomia at E18.5, body weight and body composition in adult offspring of high fat fed dams were similar to offspring of normal fat fed dams. Offspring of high fat fed dams also had normal glucose tolerance in adulthood, but did display mild fasting hyperglycaemia at 9 months of age, which may suggest skeletal insulin resistance or pancreatic dysfunction. Renal function and morphology were also similar in the two dietary groups.

Together, these outcomes indicate that metabolic health and renal function are largely unaffected in offspring exposed to maternal high fat feeding. As 9 month old mice are representative of middle-age

in humans, further studies should be performed in aged offspring. Additional studies should also incorporate a high fat feeding challenge in adult offspring, as a chronic stressor may reveal a protective effect of high nephron endowment on renal function and glomerular morphology. Alternatively, a chronic insult may reveal a reduced functional renal reserve in offspring of high fat fed dams that manifests following metabolic stress.

6.2 *Lepr*$^{db/+}$ mouse model of pre-gestational Type 2 diabetes

Preliminary studies in +/+ *misty* and *Lepr*$^{db/+}$ *misty* dams revealed normal gestational weight gain, food intake and glucose tolerance. Given the undesirable and confounding effects of *misty* on growth traits, subsequent experiments were performed following the genetic removal of *misty* from the colony. It is important to highlight that the failure of many previous studies to account for the confounding effects of *misty* on growth traits has likely led to the publication of many misleading reports. Unfortunately, removing *misty* did not improve the penetrance of glucose intolerance in *Lepr*$^{db/+}$ dams. Incomplete penetrance of glucose intolerance in late gestation in this model has not been previously reported.

An additional novel finding of the present study was that glucose intolerant *Lepr*$^{db/+}$ dams were actually mildly glucose intolerant and overweight prior to pregnancy. *Lepr*$^{db/+}$ mice presented in this thesis are therefore not a model of gestational diabetes, but rather a model of pre-gestational Type 2 diabetes that is exacerbated in pregnancy. Furthermore, while previous studies of *Lepr*$^{db/+}$ mice report offspring macrosomia (Kaufmann et al., 1981, Ishizuka et al., 1999, Yamashita et al., 2001, Yamashita et al., 2003, Stanley et al., 2011), fetal overgrowth was not observed in this study. The lack of macrosomia, the reduced penetrance of glucose intolerance in late gestation and the inability to predict which *Lepr*$^{db/+}$ dam would exhibit glucose intolerance in late gestation may discourage future use of this strain. Nonetheless, *Lepr*$^{db/+}$ mice do present a valuable model of pre-gestational Type 2 diabetes, as selected glucose intolerant *Lepr*$^{db/+}$ dams shared features of the increasingly common human condition (maternal overweight, insulin resistance and glucose intolerance before and during pregnancy) and in the absence of offspring growth restriction common to STZ studies.

As mentioned, *Lepr*$^{db/+}$ mice displayed phenotypic variation in susceptibility to glucose intolerance in pregnancy. Selecting mice based on their glucose profiles is somewhat cumbersome and requires a larger number of experimental animals. However, an advantage of *Lepr*$^{db/+}$ dams exhibiting incomplete penetrance of glucose intolerance in late gestation is the opportunity to assess nephron number in offspring of *Lepr*$^{db/+}$ dams with normal glucose tolerance. This enabled the effects of maternal genotype to be isolated from the effects of maternal glucose.

6.2.1 Decreased nephron endowment in offspring of *Lepr*$^{db/+}$ dams

Offspring of glucose intolerance *Lepr*$^{db/+}$ dams had a deficit in nephron endowment. This deficit was established before birth at a relatively early time point in nephrogenesis (E18.5) when approximately 10% of glomeruli detectable by PNA histochemistry had formed (developmentally at the S-shaped body stage or beyond). The proportionate deficit in nephron number in offspring of *Lepr*$^{db/+}$ dams at E18.5 and PN21 indicates that perturbations in early renal development are permanent and cannot be recovered post birth, despite an exponential increase in nephrogenesis during this period and despite normal glucose tolerance in *Lepr*$^{db/+}$ dams in the postnatal period. This has an important implication if the findings of the *Lepr*$^{db/+}$ study are extrapolated to women with undiagnosed pre-existing Type 2 diabetes. These women may not be identified until 24-28 weeks of gestation during routine testing for gestational diabetes. By this time ureteric branching morphogenesis and arcade formation is complete and therefore any deficit in nephron formation would already be present. This highlights the importance of an optimal intrauterine environment throughout kidney development, but especially during the relatively early stages of nephron induction and formation.

6.2.2 Potential mechanisms of altered nephron endowment in offspring of *Lepr*$^{db/+}$ mice

Unfortunately, ureteric branching morphogenesis was not assessed in offspring of +/+ or *Lepr*$^{db/+}$ dams. Based on the literature, it was expected that *Lepr*$^{db/+}$ dams would model gestational diabetes and demonstrate glucose intolerance beginning in mid-late gestation. Moreover, *Lepr*$^{db/+}$ dams were defined as glucose tolerant or glucose intolerant based on the GTT at E17.5, and thus unfortunately in this study offspring kidney development was assessed at only one embryonic time point, E18.5. Given the identified pre-gestational glucose intolerance and previous studies that report a combined deficit in ureteric branching morphogenesis and nephron endowment (Amri et al., 1999, Hokke et al., 2013), it is expected that ureteric tip number would be reduced in offspring of glucose intolerant *Lepr*$^{db/+}$ dams. Future studies should incorporate a GTT and tissue collection at an earlier time point (such as E15.5) to assess ureteric branching morphogenesis in this model.

Analysis of the molecular regulation of kidney development was beyond the scope of the present study. However, E18.5 kidneys were collected from a cohort of +/+ and *Lepr*$^{db/+}$ dams and frozen and stored for future analysis. As suggested for embryonic kidneys of high fat and normal fat fed dams, real time PCR, Western blot and immunohistochemistry could be employed to quantify and localise the expression of growth factors in kidneys of +/+ and *Lepr*$^{db/+}$ dams. As *Lepr*$^{db/+}$ offspring were hyperinsulinaemic, it would be potentially valuable to assess expression of the insulin signalling pathway in a hyperglycaemic and hyperinsulinaemic environment associated with a deficit in nephron number, and compare the findings to that found in maternal high fat feeding. Gene expression and

protein studies could also incorporate markers of ureteric tree development and nephron progenitor cell populations, and assess whether they correlate with a deficit in nephron number in offspring of *Lepr*$^{db/+}$ dams. Comparison of these factors, as well as cell proliferation signals and apoptotic markers, between kidneys of offspring of high fat fed dams and *Lepr*$^{db/+}$ dams would further our understanding of how maternal high fat feeding accelerates nephron formation and how maternal glucose intolerance is detrimental to nephron formation. Analysis of placental structure and nutrient transfer may also be valuable considering the increase in placental weight and reduction in fetal: placental weight ratio observed in *Lepr*$^{db/+}$ dams at E18.5.

6.2.3 Metabolic health and renal function in adult offspring of *Lepr*$^{db/+}$ dams

Time-constraints meant that long-term studies in adult offspring were beyond the scope of the present thesis. Follow-up studies assessing metabolic health and renal function in 6 month old offspring of +/+ and *Lepr*$^{db/+}$ dams have been performed by Nicole Arias, an Honours student in the Bertram Laboratory. In brief, adult offspring of pre-gestational diabetic dams failed to demonstrate any long-term consequences on body weight, body composition or glucose handling. Renal function and albumin excretion were also similar in the two groups. However, total glomerular volume was elevated in 6 month offspring of *Lepr*$^{db/+}$ dams despite a nephron deficit, suggesting compensatory hypertrophy of the glomeruli and the glomerular filtration surface area in order to maintain normal renal function. A manuscript detailing the experiments performed in adult offspring as well as the experiments described in Chapter 4 of the present thesis will be submitted for publication in late 2015.

6.3 Kidney development in murine models of maternal diabetes

While both high fat fed dams and *Lepr*$^{db/+}$ dams are considered models of pre-gestational glucose intolerance, the opposing effects of these two models on offspring nephron endowment are reflective of differences in maternal phenotypes. Maternal characteristics and offspring growth and kidney development in the two models are compared in **Table 6.1**. As mentioned above, increased nephrogenesis in offspring of high fat fed dams was observed regardless of glucose profiles prior to pregnancy. We propose the augmented nephron number was due to constituents of the high fat diet promoting nephrogenesis. Conversely, the decrease in nephrogenesis in offspring of *Lepr*$^{db/+}$ dams was strongly associated with maternal glucose intolerance as well as maternal overweight in late gestation. Additionally, offspring of glucose tolerant *Lepr*$^{db/+}$ dams had normal nephron endowment, further confirming that the nephron deficit in offspring of glucose intolerant *Lepr*$^{db/+}$ dams was due to maternal glucose intolerance and reinforcing this model as a suitable model of pre-gestational diabetes.

Table 6.1 Comparing high fat fed mice and *Lepr*$^{db/+}$ mice

	High fat diet vs. Normal fat diet	***Lepr*$^{db/+}$ vs. +/+**
Model of:	Pre-gestational Type 2 diabetes	Pre-gestational Type 2 diabetes
MATERNAL PHENOTYPE		
Glucose intolerance pre-pregnancy	39% ↑	18% ↑
Body weight pre-pregnancy	15% ↑	17% ↑
Glucose intolerance late pregnancy	↔	36% ↑
Body weight late pregnancy	E15.5 8% ↑ E18.5 ↔	18% ↑
OFFSPRING PHENOTYPE		
Body weight	E15.5 ↔ E18.5 5% ↑ PN21 ↔	E18.5 ↔ PN21 ↔
Ureteric branching	E15.5 ↔ E18.5 **Unknown**	**Unknown**
Nephron number	E15.5 ↔ E18.5 25% ↑ PN21 20% ↑	E18.5 17% ↓ PN21 15% ↓
Maternal factors associated with offspring nephron endowment	Late gestation glucose intolerance **No** Late gestation overweight **No** High fat diet components **Unknown**	Late gestation glucose intolerance **Yes** Late gestation overweight **Yes** Maternal genotype **No**

↑ Indicates a statistically significant increase, ↓ indicates a statistically significant decrease, ↔ indicates no statistical difference.

Comparisons can also be made between studies of maternal diabetes modelled in *Lepr*$^{db/+}$ dams and STZ-treated dams (**Figure 6.1**). Studies in offspring of STZ-induced Type 1 diabetes report a 15-40% deficit in nephron number, with diabetic pregnancy impairing nephrogenesis in proportion to maternal fasting hyperglycaemia and offspring growth restriction (Amri et al., 1999, Tran et al., 2008, Hokke et al., 2013). The similar nephron deficit reported in offspring of *Lepr*$^{db/+}$ dams and normal weight offspring of STZ-treated dams (Amri et al., 1999) indicates that intermittent episodes of postprandial hyperglycaemia may be as damaging to the developing kidney as marked fasting hyperglycaemia. As a number of women with Type 2 diabetes may be undiagnosed, this further highlights the importance of identifying glucose intolerance in women in early gestation.

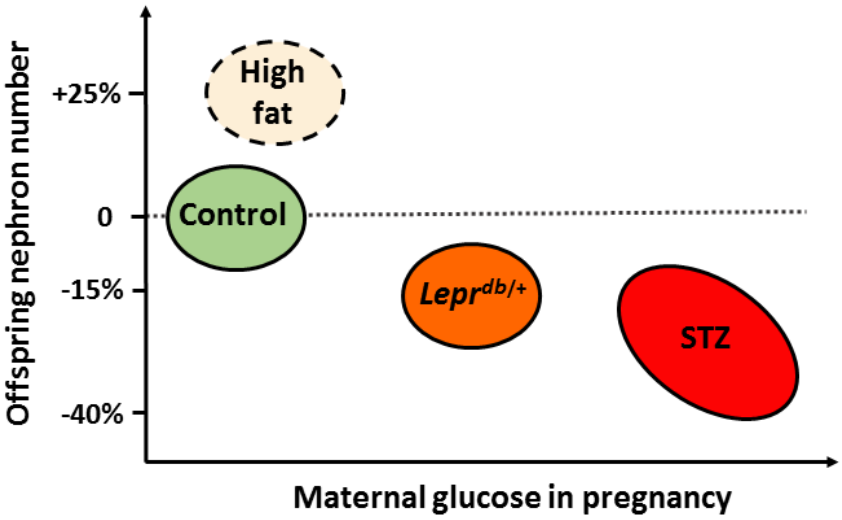

Figure 6.1 Offspring nephron number in murine models of maternal diabetes
A schematic diagram highlighting the deficits in offspring nephron number reported in murine models of pre-gestational Type 2 diabetes (*Lepr*$^{db/+}$) and Type 1 diabetes (STZ). Together, these studies highlight that an increase in maternal glucose during kidney development (whether intermittent episodes of postprandial hyperglycaemia seen in *Lepr*$^{db/+}$ dams or severe fasting hyperglycaemia seen in STZ-treated dams) is detrimental to nephrogenesis. The present study found that maternal high fat feeding increases offspring nephron number. High fat fed dams were glucose intolerant prior to pregnancy but not in late gestation. It is not known whether high fat fed dams were glucose intolerant during early kidney development.

6.4 Other animal models of diabetes in pregnancy

The studies presented in this thesis highlight the difficulty in modelling diabetes in pregnancy in mice and question the suitability of the mouse as an appropriate model. The limitations of STZ-induced diabetes have been described throughout this thesis. Continuous glucose infusion techniques are reported to model mild gestational diabetes yet lack the associated metabolic phenotypes of obesity and insulin resistance. Such methods are also technically demanding as they require the surgical

implantation of indwelling catheters. Rodent diets high in simple sugars may be a more relevant model of glucose intolerance in pregnancy in which to examine offspring kidney development. Previously, Samuelsson et al. (2013) fed C57BL6 mice a high sucrose diet for 6 weeks prior to pregnancy. While glucose and insulin profiles were not assessed in late gestation, mice displayed increased body weight prior to pregnancy, hyperinsulinaemia at weaning and fetal macrosomia. Further characterisation of this model is warranted.

An additional limitation of rodent studies investigating offspring kidney development is the discrepancy in the timing of nephrogenesis in rodents and humans. Nephron formation in humans is complete before birth at approximately 34-36 weeks gestation (Hinchliffe et al., 1991). Conversely, nephrogenesis continues into the postnatal period in mice and rats with the majority of nephrons formed after birth (Moritz and Wintour, 1999). In rodent studies assessing the effect of a maternal insult, the intrauterine environment is likely to be very different to the environment a pup is exposed to during lactation. Indeed, glucose profiles in early and late lactation were not elevated in *Lepr*$^{db/+}$ dams in the present study despite being elevated prior to pregnancy and in late gestation compared with +/+ dams.

More relevant model organisms in which to assess offspring kidney development are the sheep or Spiny mouse (*Acomys cahirinus*) as nephron endowment is complete before birth in these species (Moritz and Wintour, 1999, Dickinson et al., 2005). Diabetes in pregnancy has been modelled in ewes by STZ treatment in mid-gestation, giving rise to glucose intolerance, fetal hyperglycaemia and fetal overgrowth (Dickinson et al., 1991). Hyperglycaemia, hyperinsulinaemia and obesity can also be induced in pregnant ewes by overnutrition (Long et al., 2010, Wang et al., 2010). While pregnancy in the ewe may better mimic that of the human, the use of sheep as an experimental model is limited due to their expense, large animal husbandry requirements and long gestation time (~150 days). Spiny mice are an ideal rodent model for perinatal research as they have a relatively long gestation (~40 days), small litter sizes and precocial pups (Young, 1976). Spiny mice fed a standard rodent diet supplemented with fat-rich seeds model Type 2 diabetes characterised by obesity and glucose intolerance, as well as β cell hypertrophy and hyperplasia (Shafrir et al., 2006). Studies characterising glucose profiles in pregnant Spiny mice are sparse but offer the potential of being a valuable model in which to study fetal kidney development in maternal diabetes.

6.5 Human fetal kidney volume in gestational diabetes

Currently, estimation of total nephron number in humans is only feasible from autopsy tissue. Kidney volume measured by ultrasound, as a proxy of renal sufficiency, has been used to investigate kidney growth and development in offspring of women with Type 2 diabetes and/or gestational diabetes, yet

with equivocal findings (Verburg et al., 2007, Cappuccini et al., 2013, Neves et al., 2013). Given the paucity of human studies and the limitations of rodent studies investigating kidney development in maternal diabetes, an additional aim of the present thesis was to assess, using human obstetric ultrasonography, whether gestational diabetes in pregnancy affects fetal kidney size and volume as a measure of renal sufficiency.

Rodent studies that utilise STZ to induce maternal diabetes are commonly associated with offspring growth restriction. This is in contrast to the clinical situation, where most women with Type 1 diabetes, Type 2 diabetes or gestational diabetes are at an increased risk of fetal and neonatal macrosomia (Pedersen, 1954, Nold and Georgieff, 2004). Paradoxically, lower EFW and lower birth weight were observed in the cohort of 36 women with gestational diabetes presented in this thesis. Reduced fetal middle cerebral artery pulsatility index and amniotic fluid index were also observed in women with gestational diabetes, although these values were within the normal clinical range. Despite these outcomes, fetal kidney volume in women with gestational diabetes at 32-34 weeks gestation was similar to offspring of control pregnancies. Interestingly, fetuses of women with gestational diabetes displayed increased kidney length and depth after adjusting for EFW, indicating that fetal kidney size was unaffected despite gestational diabetes adversely affecting fetal weight and abdominal circumference.

6.5.1 Strengths, limitations and future directions of the human study

The data presented in this thesis provides a unique and comprehensive analysis of a range of maternal and fetal factors that may affect fetal renal size and volume. The present thesis describes maternal glucose profiles, maternal demographics, fetal biometry, fetal wellbeing and birth outcomes in women with and without gestational diabetes, and is distinct from previous studies, which failed to consider these parameters (Verburg et al., 2007, Neves et al., 2013).

It is recognised that the human data presented in this thesis is preliminary and part of an ongoing study. A larger study population is required to confirm the current findings and incorporate the effects of maternal ethnicity on fetal growth and renal volume in gestational diabetes, as ethnicity may have variable effects on maternal weight and BMI, maternal glucose profiles and fetal biometry. Moreover, a larger study population may identify maternal and fetal outcomes that are limited to, or exacerbated in, women of a specific ethnicity. Final data analysis will also include women diagnosed with gestational diabetes in early pregnancy, as well as women with Type 1 and Type 2 diabetes in pregnancy, to elucidate the effect of all types of maternal diabetes on human kidney development.

It should be noted that this study likely presents a low-risk group of diabetic women. These women were, in essence, self-selected as they volunteered to participate in the study. This is an important

consideration as these women were likely to be well motivated, as is evidenced by the fact that the majority of women demonstrated good glycaemic control. While it would be valuable to assess fetal kidney development in a wider, less selected cohort, women with poor glycaemic control may be difficult to capture in a research study and are likely to be a minority. To address this, final data analysis will categorise women as having mild or severe gestational diabetes based on glucose profiles and insulin requirements, thus enabling the assessment of more severe forms of gestational diabetes on fetal growth and renal development. An additional limitation of this study is the lack of data for maternal weight gain in pregnancy, as this data would prove valuable in the assessment of fetal growth.

The preliminary findings from this prospective cohort study suggest that hyperglycaemia does not affect fetal kidney volume. In humans, renal volume is proportional to renal mass and nephron endowment in the fetus and infant and is a surrogate measure of renal sufficiency (Nyengaard and Bendtsen, 1992, Zhang et al., 2008). The effect of gestational diabetes on human nephron number is unknown, however, the similar kidney sizes observed may suggest that fetal nephron number is unaffected in offspring of gestational diabetic women with good glycaemic control. Assessment of fetal kidney volume in women with longer periods of hyperglycaemia (such as women diagnosed with gestational diabetes early in pregnancy and in women identified with gestational diabetes at routine testing who are then diagnosed with Type 2 diabetes in the early post-partum period) is warranted.

6.6 Conclusions

This thesis assessed the suitability of two mouse models of glucose intolerance in pregnancy. C57BL6 mice fed a high fat diet were investigated as a potential model of pre-gestational Type 2 diabetes, yet did not exhibit glucose intolerance or obesity in late gestation. The high fat fed dams presented in this study therefore do not represent an appropriate model of maternal glucose intolerance. The augmented nephron endowment observed in offspring of high fat fed dams can instead be attributed to maternal fat feeding. *Lepr*$^{db/+}$ mice were investigated as a model of gestational diabetes, yet displayed glucose intolerance prior to pregnancy as well as in late gestation. *Lepr*$^{db/+}$ mice were therefore not a suitable model of gestational diabetes but do provide a model of pre-gestational Type 2 diabetes. Offspring of *Lepr*$^{db/+}$ dams were found to have a deficit in nephron endowment which, in conjunction with previous studies in STZ-induced diabetic dams, highlights the detrimental effect of maternal hyperglycaemia on the developing kidneys. This thesis also assessed fetal kidney volume by obstetric ultrasound in women with gestational diabetes. Preliminary data revealed normal fetal kidney size at 32-34 weeks gestation, however a larger study population is required to confirm these findings.

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