VASCULAR FUNCTION IN HYPERGLYCAEMIC PREGNANCY:STUDIES INTO POTENTIAL MECHANISMS FOR ADVERSE OBSTETRIC OUTCOMES IN DIABETES

Dr Sharon Tracey Mackin
MBChB (Hons), MRCP

Thesis submitted in fulfilment of the requirements for the degree of Doctor of Medicine (MD) at the Institute of Cardiovascular and Medical Sciences, University of Glasgow.

Abstract

Diabetes in pregnancy confers increased risk of adverse outcomes in pregnancy. Increased risks of fetal overgrowth with subsequent delivery complications and neonatal hypoglycaemia are consequence of fetal hyperinsulinaemia due to maternal hyperglycaemia. Other complications such as stillbirth and preeclampsia, both of which are increased in diabetic pregnancy are less well understood. Understanding of pathophysiological mechanisms in stillbirth and preeclampsia remain limited, but both have links to vascular dysfunction. Specifically, many cases of stillbirth are associated with features of placental vasculopathy. Preeclampsia also originates from abnormal placental vascular development, resulting in release of pro-inflammatory and angiogenic factors from the placenta into maternal circulation and subsequent endothelial and organ dysfunction. Maternal endothelial dysfunction has also been shown to exist in women prior to the onset of preeclampsia. In diabetes, the exact triggers for preeclampsia and stillbirth development are unclear. Most of our understanding of these conditions comes from studies in the general obstetric population. With preeclampsia there is a proven linear relationship with higher maternal glycaemia and increased risk of developing the condition. The hypothesis for this thesis was that diabetes in pregnancy is associated with maternal and placental vascular dysfunction as a potential mechanism for adverse outcome in pregnancy complicated by diabetes. Furthermore, it was hypothesised that such vascular dysfunction is associated with higher maternal glycaemia.

To investigate this hypothesis, I first sought to establish the prevalence of adverse outcomes in pregnancy complicated by diabetes by examining Scottish epidemiological data, and compare these to the rates seen in the population without diabetes. Between the years of 1998-2013, pregnancies complicated by type 1 or type 2 diabetes had 3-6-fold higher rates of adverse outcomes in pregnancy compared to women without diabetes. Specifically, higher rates of large for gestational age birthweight infants (52%, 38% and 10% in type 1, type 2 and no diabetes respectively), operative delivery (68% in type 1, 60% in type 2, 26% in those without diabetes) and preterm delivery (35% type 1, 22% type 2 and

6% in those without diabetes). Stillbirth rates were high at 19.5 and 24.8 per 1,000 births in type 1 and type 2, which was significantly higher than the 4.9 per 1,000 births in the population without diabetes. Worryingly, trend data showed that stillbirth and perinatal mortality data were not improving with time.

To better understand why we were failing to improve upon stillbirth statistics, I sought to identify the maternal and neonatal phenotype associated with stillbirth. I explored associations of maternal and neonatal demographics and maternal glycaemic data with stillbirth outcome in the Scottish obstetric population with diabetes. Glycaemia had a significant association with stillbirth in both type 1 and type 2 diabetes. In women with type 1 diabetes, higher HbA1c in later pregnancy had the strongest association (OR 1.06 [95% CI 1.04,1.08]) whilst in type 2 diabetes, prepregnancy HbA1c was associated (OR 1.03 [95%CI 1.00-1.04]). Maternal BMI was also linked with risk in type 2 diabetes (OR 1.07 [95% CI 1.01, 1.15]). Taken together, this suggests metabolic factors, and specifically glycaemia play a key role in stillbirth pathophysiology. Other non-metabolic risk factors identified include extremes of birthweight centiles and advancing gestation towards term both of which are linked with placental vascular insufficiency and failure.

I then performed mechanistic studies to determine differences in vascular function in pregnancies complicated by diabetes. I designed and recruited to a prospective cohort study comparing maternal and placental vascular function in women with gestational diabetes and pregnant controls with and without risk factors for GDM. For the maternal study, I was interested to see if maternal endothelial dysfunction was present in women with gestational diabetes (GDM), similar to that seen in women who later go on to develop preeclampsia. I made serial measurements of flow-mediated dilatation (FMD) of the brachial artery (a widely accepted, non-invasive test of endothelial function) from 24 weeks across the third trimester of pregnancy. FMD brachial artery was no different in women with GDM compared to both control groups at study entry (14.3+4.5% in GDM, 16.2+5.2% in risk factor group and 14.4+4.5% in control group). FMD did not change significantly with advancing gestational age, and remained no

different between groups. There was no association with biochemical measures of glycaemia or insulin resistance and FMD. This suggests that maternal endothelial function is not significantly disrupted in otherwise uncomplicated GDM pregnancy.

From the same cohort, I then went onto perform wire myography studies on chorionic plate arteries (CPAs) taken from the fetal side of placental circulation. I tested vasoreactivity to various agonists: U46619 thromboxane mimetic for contraction, calcitonin gene-related peptide to test endothelium-dependent and endothelium-independent vasodilatation mechanisms and sodium nitroprusside (SNP) to test endothelium-independent vasodilatation. In these studies, CPAs from GDM placentas showed reduced vasodilatory response to SNP with lower E_{max} (49.5+11.0% in GDM compared to 73.9+11.0% and 74.0+12.0% in risk factor and control). There was no difference in response to the other agonists tested suggesting that cyclic GMP (cGMP) pathways are altered in GDM pregnancy. Furthermore, there was significant inverse correlation with fasting blood glucose (Pearson's r -0.526, p=0.01) and significant effect of higher glucose on lower E_{max} to SNP (p=0.03). In multivariate modelling, insulin resistance measures were not associated with SNP E_{max}.

In summary, adverse obstetric outcomes are high in the population with diabetes and are significantly so compared to the population without diabetes. Despite best efforts of clinical teams and mothers, serious outcomes such as stillbirth and perinatal mortality are not improving. Glycaemia is an important modifiable risk factor associated with fetal mortality. Placental vascular vasodilatory response is reduced in GDM pregnancy. Specifically, this appears specific to altered cGMP pathways and is negatively associated with higher fasting blood glucose. This could be a possible mechanism for placental vascular dysfunction and adverse outcome in diabetic pregnancy, particularly where glycaemia is uncontrolled. Conversely, maternal endothelial function did not appear significantly altered by GDM suggesting that this is not a major mechanism to the higher rates of adverse outcome in such pregnancies. Further mechanistic work

is required to better understand effects of glucose on the fetal side of the placental circulation.

Table of Contents

List of Tables

List of Figures

Publications and presentations related to work in this thesis

Publications

Lindsay RS, *Mackin ST*, Nelson SM. Gestational diabetes mellitus – right person, right treatment, right time? BMC Medicine 2017;15(1):163

Mackin ST, Nelson SM, Kerssens JJ et al. Diabetes and pregnancy: national trends over a 15 year period. Diabetologia 2018;61(5):1081-1088

Mackin ST, Nelson SM, Wild SH et al. Factors associated with stillbirth in women with diabetes. Diabetologia 2019;62(10)1938-1947

Dobbie LJ, *Mackin ST*, Hogarth K et al. Validation of semi-automated flow-mediated dilatation measurement in healthy volunteers. Blood Pressure Monitoring 2020:216-213

Oral presentations

Gestational diabetes and altered placental vascular function - Yorkhill Paediatric Research Day (Glasgow), November 2019

Gestational diabetes and altered placental vascular function - European Diabetes and Pregnancy Study Group (Graz, Austria), September 2019

Diabetes and pregnancy outcomes: mechanisms of disease - NHS Research Network Diabetes Research Day (Glasgow), May 2019

Predictors of stillbirth in women with pregestational diabetes: national outcomes - Caledonian Society of Endocrinology and Diabetes (Dunkeld), Feb 2019

Determinants of stillbirth in women with pregestational diabetes – Yorkhill Paediatric Research Day (Glasgow) – November 2018

Stillbirth in pregestational diabetes: defining associated maternal and neonatal characteristics in the Scottish population – European Diabetes and Pregnancy Study group (Rome), September 2018

Stillbirth in pregestational diabetes: defining associated maternal and neonatal characteristics in the Scottish population - Scottish Diabetes Group Pregnancy subgroup, Scottish Government (Edinburgh), September 2018

Diabetes and pregnancy in Scotland: 15 year observational outcome data from 1998-2013 - Diabetes UK Professional Conference (London) March 2018

Type 1 and type 2 diabetes: tracking change in Scottish perinatal outcomes - European Diabetes and Pregnancy Study Group (Nyborg), September 2017

Awards

Diabetes and Pregnancy Study Group Research Travel award, September 2019

Caledonian Society of Endocrinology and Diabetes Research prize, 2019

Yorkhill Prize for Postgraduate Research, 2018 (Second prize)

Diabetes and Pregnancy Study Group Research Travel award, September 2018

Diabetes and Pregnancy Study Group Research Travel award, September 2017

Acknowledgement

The work in this thesis would not have been possible without the support and contributions from a variety of people.

Firstly, I would likely to thank the patients who contributed to this study. Pregnancy is such a busy time with numerous antenatal visits scheduled, so to participate in this study despite this is remarkably selfless and for that I will be forever grateful.

I wish to extend a huge thank you to my supervisors Dr Robbie Lindsay and Professor Christian Delles. Dr Robbie Lindsay, you have consistently championed for me in both academia and clinical medicine across many years. Unfazed by my many instances of imposter syndrome, you have continued to believe in me and push me forward for huge opportunities that will allow me to become the expert in diabetes and pregnancy. You also supported my application for consultancy, meaning your efforts have now landed yourself with me as a colleague forever. Thank you for your unwavering mentorship. Professor Christian Delles, you have been equally transformative as a mentor. You have been completely unfazed by my stupid questions about "science," and despite my novice skills have entrusted me with collaborations with some of your most renowned colleagues. You have encouraged me to think like a clinician scientist, and in my opinion, achieved the impossible with this. Thank you for your steadfast patience (some might call it endurance) and guidance. Despite the murky waters of the River Clyde separating our clinical workplaces, I hope that we can continue to work together in the future.

Thank you also to my funder, the Glasgow Children's Hospital Charity. Thank you for choosing me and this project for funding. Without this, I may not have been able to undertake this work. I hope you see it as a valuable contribution to women and child's health sciences.

In addition, I would like to thank Dr Dilys Freeman and her lab and Dr Delyth Graham for their assistance in preparing me to begin my placental studies. From simple things like loaning me your placental biopsy kit, to allowing me access to placental tissue to practice myography on. A specific thank you to Dr Freeman for putting me in touch with Dr Mark Wareing at the University of Manchester so that I could visit their specialist placental myography unit. Dr Mark Wareing and colleagues in the University of Manchester taught me so much in a short space of time. Without this, I would have taken much longer to get started with my study and almost certainly would have ran into difficulties with lengthy protocol development. I am very grateful to you all for sharing your expertise. Mrs Laura Haddow and staff, you first introduced me to the wire myography suite and spent several days and hours training me at the CORE facility in the University of Glasgow. Your training, advice and entertaining chat made the long hours in myography much more pleasant.

Further thanks are extended to the NHS Greater Glasgow and Clyde research midwifery team for their input in this work. Therese McSorley, Lorna McKay, Kirsteen Paterson, Nadia Palma and Isobel Crawford in particular assisted significantly with my recruitment. I am certain that without their input, I would have found recruitment more challenging.

In addition, I am grateful to my clinical and academic colleagues in the Scottish Diabetes Research Network Epidemiology subgroup. Feedback on my studies, strengthened my study and am glad that we get to continue to collaborate over the next few years.

To my pals in lab 313, you are all awesome and I am so lucky to have been grouped with you. We shared many good times with laughter, babies and cake, bad times with broken ankles (plural), frustration and tears and even some serious times where we did work. Dr Angela-Lucas Herald, Dr Eilidh McGinnigle, Dr Daniele Ker, Dr Giacomo Rossito, Dr Katriona Brooksbank and Dr Sheon Samji, I could not have done it without you. Viva Team 313!

To my now forever colleagues in Glasgow Royal Infirmary Department of Diabetes, Endocrinology and Clinical Pharmacology, thank you for your many pep talks over the last year or so. I finally did manage to “just get the thesis done.”

Finally, a massive thank you to my family. To my husband Paul, the last few years have not been easy for you. Having started off with a wife with an already highly-strung baseline, the love and tolerance you have shown me over the past few years has been nothing short of admirable. Thank you for sticking in there! To my mum, Patricia – having already “done your time” bringing up four young children, you have uncomplainingly taken on more years of often unscheduled childcare with my two boys. Thank you for being my rock. And to my two handsome boys, Owen (age 7) and Lewis (age 5). Thank you for always making me smile. You are my biggest achievement and I love you very much.

Author's Declaration

This thesis was composed entirely by the author, Dr Sharon Mackin. I confirm that no part of this thesis or work has been previously submitted in support of another degree.

The author (under supervision of Professor Christian Delles and Dr Robbie Lindsay) was responsible for: study design, obtaining ethics and R&D approvals, development and maintenance of study paperwork, recruitment, conducting all study visits and procedures at these visits including maternal vascular phenotyping, sample collection, all aspects of myography experiments, biochemical assay analysis, data collection and statistical analysis. A number of others also contributed to the work within this thesis. For the mechanistic studies contained in chapters 5 and 6, all statistical analysis was conducted by the author but for the work in chapters 3 and 4, statistical support was provided. The epidemiological work contained within chapter 3 benefited from input from Scottish Diabetes Research Network statistician Dr Joannes Kerssens. For these studies, the author was responsible for development of study question, study design including development of pre-defined analysis plans, interpretation of statistical outputs provided by Dr Kerssens and review of analysis following peer review. The author also contributed directly to analysis of trend data in this chapter. In chapter 4, the author and supervisor Dr Robbie Lindsay conducted statistical analysis jointly. The NHS Greater Glasgow and Clyde research midwife team led by senior midwife Therese McSorley helped with recruitment of participants from NHS Greater Glasgow and Clyde obstetric clinics. Biochemical analysis of glucose, lipids, HbA1c and fructosamine was performed by Elaine Butler on the Cobas autoanalyser, with assistance from the author during this process. Assays performed on the Luminex™ platform were conducted by the author.

Dr Sharon Mackin

July 2021

Definitions/Abbreviations

ACOG	American College of Obstetricians and Gynaecologists
ACE	Angiotensin converting enzyme
ADMA	Asymmetric dimethylarginine
AGE	Advanced glycation end products
AT_1R	Angiotensin II receptor type 1
AT_2R	Angiotensin II receptor type 2
AUC	Area under curve
BMI	Body Mass Index
BP	Blood Pressure
cAMP	Cyclic adenosine monophosphate
CGM	Continuous glucose monitoring
cGMP	Cyclic guanosine monophosphate
CGRP	Calcitonin gene related peptide
CHI	Community health index
COX	Cyclooxygenase
CPA	Chorionic plate artery
CCRC	Cumulative concentration response curve

CS	Caesarean Section
CSO	Chief Scientist's Office
DBP	Diastolic blood pressure
DHEA	Dehydroepiandrosterone
DIP	Diabetes in pregnancy
DOC	Deoxycorticosterone
E2	Estradiol
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
ETA	Endothelin receptor A
ETB	Endothelin receptor B
FIGO	International Federation of Obstetricians and Gynaecologists
FPG	Fasting plasma glucose
GC	Guanylyl cyclase
GDM	Gestational Diabetes Mellitus
GH	Growth hormone
HAPO	Hyperglycaemia and Adverse Pregnancy Outcomes
HbA1c	Glycated haemoglobin A1c

hCG	Human chorionic gonadotropin
HDL	High density lipoprotein
HELLP	Haemolysis, elevated liver enzymes and low platelets
HOMA-IR	Homeostatic model assessment of insulin resistance
hPL	Human placental lactogen
HUVEC	Human umbilical vein endothelial cell
IADPSG	International Association of Diabetes and Pregnancy Study Group
IGF	Insulin-like growth factor
IQR	Interquartile Range
IL-	Interleukin
iNOS	Inducible nitric oxide synthase
IOM	Institute of Medicine
ISD	Information Services Division Scotland
ISSHP	International Society for the Study of Hypertension in Pregnancy
IUGR	Intrauterine growth restriction
K_{Ca}	Calcium gated potassium channel
kPa	kilopascals
KDR	Kinase insert domain receptor

KPSS	Potassium containing physiological salt solution
LGA	Large for gestational age
LMWH	Low molecular weight heparin
LNAME	N(ω)-nitro-L-arginine methyl ester
MAPK	Mitogen activated protein kinase
MLCK	Myosin light chain kinase
mmHg	Millimetres of mercury
NDDG	National Diabetes Data Group
NICE	National Institute for Health and Care Excellence
NICU	Neonatal intensive care unit
NO	Nitric oxide
NOS	Nitic oxide synthase
NPID	National Pregnancy in Diabetes
NPV	Negative predictive value
O_2^-	Superoxide
OGTT	Oral glucose tolerance test
$ONOO^-$	Peroxynitrite
PDE	Phosphodiesterase

PGDM	Pregestational diabetes mellitus
PGI_2	Prostacyclin
pGH	Placental growth hormone
PIH	Pregnancy induced hypertension
PKG	Protein kinase G
PPV	Positive predictive value
PSS	Physiological salt solution
RAAS	Renin angiotensin aldosterone system
RAGE	Receptor for advanced glycation end products
RCOG	Royal College of Obstetricians and Gynaecologists
RCT	Randomised control trial
RDS	Respiratory distress syndrome
ROS	Reactive oxygen species
SBP	Systolic BP
SDRN	Scottish Diabetes Research Network
sFlt-1	Soluble fms-like tyrosine kinase-1
SGA	Small for gestational age
SIMD	Scottish index of multiple deprivation

SMR02	Scottish Maternity Record 02
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
T1DM	Type 1 diabetes
T2DM	Type 2 diabetes
Th1	T-helper 1
Th2	T-helper 2
TIR	Time in range
TNFα	Tumour necrosis factor α
TXA_2	Thromboxane A2
VSMC	Vascular smooth muscle cell
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor 2
WHO	World Health Organisation

1 Chapter 1: Introduction

1.1 Diabetes and pregnancy

Hyperglycaemia is the most prevalent metabolic disorder complicating pregnancy. Diabetes during pregnancy can be categorised broadly into three groups: 1) Pre-gestational diabetes (PGDM) which includes women entering pregnancy with a diagnosis of chronic diabetes mellitus pre-dating pregnancy 2) diabetes in pregnancy (DIP) which includes women who have severe hyperglycaemia first detected in pregnancy that meet the standard diagnostic criteria for diabetes, and 2) gestational diabetes (GDM) i.e. women who have evidence of glucose intolerance first detected in pregnancy. DIP was first introduced as a term in the World Health Organisation (WHO) 2013 guidelines in recognition that women who have more severe diabetes were more likely to experience adverse outcomes in pregnancy, were more likely to require aggressive pharmacotherapy, were often excluded from trials looking at GDM outcomes, and were at risk of diabetes complications (World Health Organisation 2013).

DIP and PGDM, can be further subclassified according to the type of diabetes. Type 1 diabetes (T1DM) which is an autoimmune condition resulting in pancreatic ß-cell failure and an absolute insulopaenic state and type 2 diabetes (T2DM), which is associated with insulin resistance and resultant hyperglycaemia are the two most common types of diabetes within this category. Depending on longevity of diabetes, patients with T2DM diabetes usually have hyperinsulinaemia to compensate for the insulin resistance (earlier stages of disease), but can progress to ß-cell failure and absolute insulin deficiency in long-standing advanced T2DM. DIP and PGDM will also include rarer causes of pre-existing diabetes including secondary pancreatic diabetes and genetic forms of diabetes.

The International Diabetes Federation estimated around 16% of all pregnancies worldwide are complicated by hyperglycaemia. Of these, the vast majority of

have GDM (86%) whilst the remainder consist of PGDM (6%) and DIP (7%) (Cho et al., 2018). However, in the case of GDM, diagnostic criteria vary internationally and have changed with time, meaning that wide variation in prevalence is reported across different studies. Furthermore, regional variation in GDM occurs due to genetic risk associated with different ethnicities. A systematic review of UK-based studies showed regional prevalence of GDM ranging from 8-24% (Farrar D, 2016).

1.1.1 Normal glucose homeostasis in pregnancy

In order to optimise fetal growth, maternal metabolic adaptations must occur to promote nutrient delivery from mother to fetus. As such, a host of coordinated hormonal adaptations occur from early pregnancy. In early pregnancy, maternal adaptation shows an initial increase in insulin secretory response and an increase in insulin sensitivity compared to the pre-pregnancy state. This leads to increased lipogenesis and adipose stores as a fuel reserve for later pregnancy (Powe et al., 2019). As pregnancy progresses, maternal insulin signalling is altered such that insulin sensitivity decreases whilst insulin secretion and ß-cell mass increases (Powe et al., 2019, Angueira et al., 2015). This insulin resistant state coupled with increased adiposity, acts to promote maternal free fatty acid production, hepatic gluconeogenesis and ultimately increased availability of glucose to the developing fetus. Post-prandial glucose levels are typically higher in later pregnancy than in the non-pregnant state, whereas fasting levels tend to be lower.

The changes in insulin sensitivity seen in pregnancy are driven by some part in increasing levels of placental-derived hormones such as oestradiol, progesterone, placental growth hormone and lactogen, alongside altered corticosteroid metabolism. There is a continual increase in expression of these pregnancy hormones from the conceptus and placenta from early pregnancy, peaking alongside insulin resistance in the third trimester. These hormones have a multitude of effects to help support pregnancy, and their role in placental development and glucose homeostasis are discussed individually in chapter 1.2.

In GDM, this homeostasis is dysregulated in favour of pathological insulin resistance and compensatory mechanisms to combat hyperglycaemia are insufficient (Baz et al., 2016). Insulin secretion from pancreatic ß-cells is significantly reduced in the order of 30-70% in GDM mothers compared with non-GDM mothers, whereas insulin sensitivity is slightly reduced (Angueira et al., 2015). Obese mothers are more susceptible to development of GDM, with a predisposition to insulin resistance from high adiposity contributing further to insulin resistance in pregnancy (Li et al., 2014).

1.1.2 Diagnostic criteria for DIP

The diagnostic criteria used to diagnose DIP are the same as those glucose criteria used to define diabetes in the non-pregnant population, except it is first detected in pregnancy. This is any of:

- fasting plasma glucose (FPG) $\geq$ 7.0 mmol/L
- 2-hour post-75g oral glucose tolerance test (OGTT) $\geq$ 11.1 mmol/L
- random plasma glucose $\geq$ 11.1 mmol/L in the presence of symptoms of hyperglycaemia or
- HbA1c $\geq$ 48 mmol/L

These criteria are applicable across all stages of pregnancy (2013).

1.1.3 Diagnostic criteria for GDM

Diagnostic criteria for GDM are more contentious and cause debate amongst experts about which glycaemic criteria best estimate increased obstetric risk. This is partly because there is a continuous relationship with maternal glycaemia with no clear inflection point at which risk of adverse pregnancy outcome is increased (Metzger et al., 2008). Furthermore, GDM screening is utilised differently across healthcare settings, with some populations undergoing

universal screening in pregnancy, and others screening only those with identified risk factors for GDM, such as obesity and family history of diabetes.

1.1.3.1 Historical evolution of GDM diagnostic criteria

Prior to 2010, international guidelines had higher glycaemic thresholds for diagnosing GDM than current guidelines, although glycaemic markers were still significantly lower than those used to diagnose overt diabetes (table 1.1). The earliest GDM criteria using maternal whole blood glucose levels were published by O'Sullivan and Mahan in 1964, although concerns of morbidity and mortality associated with diabetes during pregnancy had been recognised before this. O'Sullivan and Mahan used statistical modelling to identify the second and third deviations of upper limit of normal of maternal glucose levels in later pregnancy post 100g-OGTT and produced criteria for GDM that included: FBG >5.0 mmol/L, 1-hour post-OGTT >9.2 mmol/L, 2-hour post-OGTT >8.1mmol/L and 3 hours post-OGTT >7.0mmol/L. Diagnosis required at least two of these parameters to be met (O'sullivan and Mahan, 1964). These criteria were originally designed to identify those women who had an increased risk of developing T2DM post-partum although there was concurrent recognition that obstetric risk was increased in these women. In later years, these criteria were further refined as laboratory techniques became more advanced and allowed better detection of glucose in plasma compared with older technologies using whole blood (National Diabetes Data Group criteria (NDDG)), and furthermore when technology advanced that could distinguish glucose from other reducing substances (Carpenter and Coustan, 1982). These refined criteria are still adopted today for GDM diagnosis in some nations including the United States of America, and are termed the Carpenter-Coustan criteria. When compared with NDDG, Carpenter-Coustan criteria have been shown to diagnose higher numbers of women with GDM, but also were superior at detection of pregnancies at risk of adverse outcome. In a large American cohort, Carpenter-Coustan criteria resulted in a 42% relative increase in women diagnosed with GDM versus NDDG criteria, but these women who would have went undetected by NDDG criteria had a 70% increased risk of

preeclampsia, 16% increased risk of operative delivery and a 25% increased risk of delivering a large birthweight infant (>4000 g) (Berggren et al., 2011).

The diagnostic criteria recommended by different prominent learned societies across time are listed in table 1.1. Not only was there debate about the glycaemic thresholds used for diagnosis, but differences in the glucose load used in OGTT were also disputed. For example, the WHO showed preference for the 75g OGTT, as there was consensus internationally that this was the optimal dose for diagnosing diabetes in the non-pregnant individual. American societies favoured the 100g OGTT from the original O'Sullivan and Mahan, due to lack of validation of the lower dose in pregnant populations when guidance was first developed (National Diabetes Data Group, 1979).

In recognition of the need for an evidenced-based international consensus on GDM diagnosis and the prediction of adverse outcomes in GDM, the landmark Hyperglycaemia and Adverse Pregnancy Outcomes (HAPO) study was conducted and published in 2008 (Metzger et al., 2008). The results and impact of this study are discussed in the next section. From the HAPO data, new diagnostic criteria where developed by the International Association of Diabetes and Pregnancy Study Group (IADPSG) (table 1.1) (International Association of Diabetes and Pregnancy Study Groups Consensus, 2010).

There remains variation in diagnostic criteria internationally. IADPSG criteria are currently used in Scotland, Australia and New Zealand, and Ireland amongst others, and are endorsed by the International Federation of Obstetricians and Gynaecologists (FIGO). The Carpenter and Coustan criteria are favoured by the American College of Obstetricians and Gynaecologists (ACOG Practice Bulletin 190, 2018). Other countries have their own variations of these based on clinical and economic analysis of outcomes and criteria in their populations. For example, the National Institute for Clinical Excellence adopted the IADPSG criteria but altered them slightly after health economic analysis in 2015 demonstrated improved cost effectiveness with FBG >5.6 mmol/L and 2-hour post-75g OGTT >7.8 mmol/L. Obstetric outcomes included in the economic

model were shoulder dystocia, caesarean section, induction of labour, preeclampsia, neonatal jaundice and neonatal intensive care stay (Diabetes in Pregnancy (National Institute for Health and Care Excellence), 2015) .

Table 1.1: Different glycaemic thresholds used for diagnosis of GDM according to international guidelines

	National Diabetes Data Group (Berggren et al., 2011)	**Carpenter and Coustan (Berggren et al., 2011)**	**World Health Organisation (WHO) 1999 (2013)**	**IADPSG 2010 and WHO 2013 (2013)**	**National Institute for Clinical Excellence (NICE) 2015 (National Collaborating Centre For and Children's, 2015)**
OGTT glucose dose	100g	100g	75g	75g	75g
Diagnostic thresholds	Fasting ≥ 5.8 mmol/L 1 hour ≥ 10.6 mmol/L 2 hour ≥ 9.2 mmol/L 3 hour ≥ 8.0 mmol/L	Fasting ≥ 5.3 mmol/L 1 hour ≥ 10.0 mmol/L 2 hour ≥ 8.6 mmol/L 3 hour ≥ 7.8 mmol/L	Fasting ≥ 7.0 mmol/L 2 hour ≥ 7.8 mmol/L	Fasting ≥ 5.1 mmol/L 1 hour ≥ 10 mmol/L 2 hour ≥ 8.5 mmol/L	Fasting ≥ 5.6 mmol/L 2 hour > 7.8 mmol/L
Number of thresholds exceeded required for diagnosis	At least 2 of above	At least 2 of above	Any one of above	Any one of above	Any one of above

1.1.3.2 Hyperglycaemia and Adverse Pregnancy Outcome (HAPO) study

The HAPO study was a large, multicentre observational study that explored the association of adverse maternal and neonatal outcomes with maternal glycaemia, and included women across the spectrum of glycaemia that was below the threshold for diagnosing DIP. In this study, women underwent a 75g OGTT between 24-32 weeks of gestation with fasting, 1-hour and 2-hour post-

OGTT plasma glucose levels measured. The primary outcome was to measure the association of maternal glycaemia with large for gestational age (LGA) infant (birthweight >90th centile), need for primary caesarean delivery, occurrence of neonatal hyperglycaemia and cord serum C-peptide level indicating neonatal hyperinsulinaemia. However, it also explored a number of important secondary maternal and neonatal outcomes. Table 1.2 shows the adjusted odds ratio for each incremental rise in maternal glycaemic parameters (Metzger et al., 2008).

Table 1.2: Hyperglycaemia and Adverse Pregnancy Outcomes (HAPO) study results of adjusted odds ratio of adverse pregnancy outcomes per incremental increase in fasting, 1-hour and 2-hour maternal plasma glucose levels post 75g GTT.

	Odds ratio per 0.4 mmol/L increase in fasting plasma glucose	Odds ratio per 1.7 mmol/L increase in 1-hour 75g OGTT plasma glucose	Odd ratio per 1.4 mmol/L increase in 2-hour 75g OGTT plasma glucose
Birthweight > 90th centile	**1.38 [1.32-1.44]**	**1.46 [1.39-1.53]**	**1.38 [1.32-1.44]**
Caesarean section	**1.10 [1.06-1.15]**	**1.10 [1.06-1.15]**	**1.08 [1.03-1.12]**
Clinical neonatal hypoglycaemia	1.08 [0.98-1.19]	**1.13 [1.03-1.26]**	1.10 [1.00-1.12]
Cord blood serum C-peptide > 90th centile	**1.55 [1.47-1.66]**	**1.46 [1.38-1.54]**	**1.37 [1.30-1.44]**
Preterm delivery < 37 weeks	1.05 [0.99-1.11]	**1.18 [1.12-1.25]**	**1.16 [1.10-1.23]**
Shoulder dystocia	**1.18 [1.04-1.33]**	**1.23 [1.09-1.38]**	**1.22 [1.09-1.37]**
Neonatal intensive care admission	0.99 [0.94-1.05]	**1.07 [1.02-1.13]**	**1.09 [1.03-1.14]**
Neonatal jaundice	1.00 [0.95-1.05]	**1.11 [1.05-1.17]**	**1.08 [1.02-1.13]**
Preeclampsia	**1.21 [1.13-1.29]**	**1.28 [1.20-1.37]**	**1.28 [1.20-1.37]**

Table adapted from table 2, Hyperglycaemia and adverse pregnancy outcome (HAPO) study, NEJM 2008 (Metzger et al., 2008). Results in bold red show those with statistically significantly elevated odds ratios.

The study showed a linear and continuous relationship with maternal glycaemia and adverse pregnancy outcomes. Smaller increments in fasting plasma glucose levels were associated with similar odds ratios seen with higher increments in post-OGTT glucose levels. Importantly, there was no clear threshold at which risk was exponentially increased, and no clear level of glycaemia which could define pregnancies as binary "high" or "low" risk (Metzger et al., 2008). Smaller observational cohort studies had previously shown similar continuous relationships with increasing maternal glycaemia and adverse outcomes (Pettitt et al., 1980, Sermer et al., 1995). HAPO methodology had several advantages over these earlier studies in confirming these findings. The HAPO study was larger, included a heterogenous group of women from various international centres and, importantly, included blinding of researchers, caregivers and participants to glycaemic measures in an attempt to limit bias (Metzger et al., 2008).

Using HAPO data, the IADPSG reached a new consensus on GDM diagnosis in 2010 (International Association of Diabetes and Pregnancy Study Groups Consensus, 2010). They agreed maternal glycaemic cut-offs for GDM diagnosis based on OGTT data that showed a 75% increased risk of primary outcome (Large for gestational age (LGA), caesarean section, neonatal hypoglycaemia, neonatal hyperinsulinaemia) compared to the mean in the HAPO cohort. The IADPSG criteria for GDM diagnosis using a 75g OGTT between 24-28 weeks are as follows:

- Fasting $\geq$ 5.1 mmol/L
- 1-hour $\geq$ 10.0 mmol/L
- 2-hour $\geq$ 8.5 mmol/L

Whilst the HAPO study examines glycaemic data from the later stages of pregnancy (24-32 weeks) where insulin resistance is known to be increasing, the IADPSG recognised that some women and populations would be at higher risk of developing GDM in earlier stages of pregnancy. They therefore agreed that,

whilst the above criteria are the gold-standard for diagnosis, clinicians can use a fasting plasma glucose in the earlier stages of pregnancy > 5.1 mmol/L (but lower than overt DIP (7.0 mmol/L)) to diagnose GDM if clinically felt to be at higher risk of GDM but its use in earlier pregnancy is controversial. In a study of women with GDM with FBG >5.1 mmol/L in early pregnancy, less than 40% met diagnostic criteria for GDM when tested between 24-28 weeks (Zhu et al., 2013, Mcintyre et al., 2016). In cases where earlier testing is adopted, it is important that clinicians follow up women who test negative for GDM in earlier pregnancy with repeat testing with the gold-standard 75g OGTT at 24-28 weeks (International Association of Diabetes and Pregnancy Study Groups Consensus, 2010).

1.1.3.3 Utilisation and comparison of IADPSG and other GDM diagnostic criteria

These new IADPSG criteria are now the most widely used criteria for GDM diagnosis, and are endorsed by leading societies such as the WHO and FIGO (World Heatlh Organisation, 2013, Hod et al., 2015). They are used routinely across Scotland (Scottish Intercollegiate Guidelines Network, 2017). However, they are not universally adopted across the UK, nor in some other countries. The main criticism of IADPSG criteria is that it leads to significantly higher numbers of women diagnosed with GDM compared with Carpenter-Coustan, which in a sceptical view leads to medicalisation of pregnancy and increased financial and resource pressures on obstetric services. For example, in the HAPO cohort, prevalence of GDM would increase from 4% if diagnosed using Carpenter-Coustan criteria but would be almost four-fold higher at 14% if the marginally tighter IADPSG criteria were used (Waters et al., 2016). This same study showed that those diagnosed according to IADPSG criteria but not by Carpenter-Coustan definitions, had significantly higher rates of adverse pregnancy outcome compared to women without GDM. Specifically, they had higher rates of fetal overgrowth as measured by birthweight centiles and neonatal adiposity measures, higher rates of neonatal hypoglycaemia, increased need for caesarean section and greater risk of preeclampsia compared to women without GDM (Waters et al., 2016). It is important to recognise that the HAPO group were an

untreated GDM group, since evidence has shown that treatment of mild GDM improves pregnancy outcomes (Landon et al., 2009, Crowther et al., 2005, Duran et al., 2014). Evidence of benefit of IADSPG versus Carpenter-Coustan criteria in real-world, non-blinded and treated GDM populations has proven more controversial. Duran et al found that adoption of IADPSG criteria in a clinical setting led to higher prevalence of GDM, but also resulted in significantly lower rates of hypertensive disorder, caesarean section, small- and large- for gestational age infants, and neonatal ICU admissions (Duran et al., 2014), and therefore were cost-effective in that population. Whilst other population surveys have shown no significant difference in prevalence of adverse obstetric outcome between IADPSG and Carpenter-Coustan (Feldman et al., 2016, Gariani et al., 2019) which raises questions about cost-effectiveness and concerns of overmedicalisation of pregnancy for women. Some societies, including ACOG have opted against recommendation of IADPSG criteria over more traditional screening.

Furthermore, some societies such as ACOG opt for a two-step approach where women are initially screened with a non-fasted 1-hour 50g glucose challenge, and only proceed to a 100g OGTT for diagnosis if the initial challenge results in plasma glucose greater than 7.1 mmol/L. Randomised control trial (RCT) data showed a doubling of GDM diagnoses in the American population using the one-step IADPSG criteria, compared to the two-step approach yet both groups had similar rates of adverse outcomes (Hillier et al., 2021). Adding further controversy, but acknowledging the important need for cost-effective screening tools in clinical practice, the National Institute of Health and Care Excellence (NICE) performed economic modelling of diagnostic thresholds for GDM and subsequent obstetric outcomes. In 2015, they published slightly different criteria (table 1.1) from the other methods in use that took this into account (National Insitute for Health and Care Excellence, 2015). Observational data from a UK population has shown that using these guidelines over IADPSG would only miss a small proportion of women (4.1% NICE vs 4.6% IADPSG prevalence), but these missed women had significantly higher risk of adverse outcome compared to the

control non-GDM group (3-fold higher risk for LGA, 7-fold higher risk for polyhydramnios, >2-fold higher for preeclampsia) (Meek et al., 2015).

In summary, the HAPO study was a landmark study that demonstrated prospectively an increase in adverse pregnancy outcomes with increasing levels of maternal hyperglycaemia, even at the milder end of the range. This led to more universal recognition that some of the diagnostic thresholds used previously were too high, with risk of missing high numbers of women who could benefit from treatment and increased surveillance that a diagnosis of GDM would afford. However, some diagnostic criteria, such as Carpenter Coustan, are only marginally different to IADPSG and the evidence from population cohorts of the benefit of the lower IADPSG thresholds is variable. Heterogeneity of treated populations in these largely retrospective studies make comparison between the two difficult, and so some expert clinical societies have struggled to derive evidence of benefit that is applicable to their population. Furthermore, all of these studies explore the risk of adverse obstetric outcomes. With time, we would hope to establish evidence to identify if any of the GDM diagnostic criteria better differentiate longer term risk of metabolic sequelae in mothers and their offspring.

1.1.4 Complications of pregnancy complicated by diabetes

Pregnancy complicated by hyperglycaemia, whether that be DIP or GDM, is associated with increased risk to the health of the mother and her offspring, both during and after pregnancy. Overall risks during pregnancy are higher in women who have pre-existing diabetes entering pregnancy, than in those with newly diagnosed GDM. However, adverse outcomes are noted with increased frequency in both groups compared to the general obstetric population.

1.1.4.1 Fetal overgrowth

Macrosomia (defined as infant birthweight >4000g or >4500g depending on the study) or LGA (>90th centile birthweight corrected for gestational age) is the most common complication of pregnancy complicated by diabetes. It is of

concern in pregnancy due to the associated risks of obstructive labour with birth injury, need for caesarean section to prevent or treat delivery complications associated with large infants, and an increased risk of neonatal hypoglycaemia (Yamamoto et al., 2017). Studies have shown that up to 50% of mothers with T1DM will deliver an LGA infant, with lower rates seen in obstetric populations with T2DM (approximately 25-30%) (Evers et al., 2002, Maresh et al., 2015, Ladfors et al., 2017). Rates are also significantly elevated in GDM with up to a third of women delivering LGA infants (Ehrenberg et al., 2004). These are significantly above the background rates of LGA, which by definition should be approximately 10% if referenced to a similar population. Fetal overgrowth has long been recognised as a problem in such pregnancies. In 1952, Pedersen hypothesised that glucose transfer across placenta resulted in fetal hyperglycaemia and hyperinsulinaemia, resulting in increased lipogenesis and accelerated fetal adiposity growth (Pedersen, 1952). Maternal glycaemia has an important association with risk of macrosomia. In women with T1DM, the risk of macrosomia was higher in those with higher post-prandial blood glucose in later stages of pregnancy (20% in mothers with post-prandial <6.7 mmol/L compared with 35% in those with post-prandial > 8.9 mmol/L) (Jovanovic-Peterson et al., 1991). Glycaemia in the second and third trimester is one of the strongest predictors of fetal overgrowth in T1DM (Maresh et al., 2015, Ladfors et al., 2017, Jovanovic-Peterson et al., 1991). Importantly, greater glycaemic variability throughout the day has been shown to have an association with LGA over and above that of measures of average glycaemia, such as HbA1c (Law et al., 2015, Scott et al., 2020). In T2DM and GDM, higher glycaemia is also associated with macrosomia, and treatment of high blood glucose is associated with improved rates of macrosomia (Crowther et al., 2005, Landon et al., 2009, Ladfors et al., 2017).

Maternal BMI is a further risk factor for LGA regardless of diabetes type or status (Kong et al., 2019, Stogianni et al., 2019). Obesity is prevalent amongst women with GDM and T2DM, but prevalence is increasing more generally worldwide and so affects many pregnancies complicated by T1DM also. Ideally, weight should be optimised before women enter pregnancy, but this is difficult for women to

achieve. Studies have shown that overweight women who reduce their weight in between pregnancies are less likely to deliver LGA infants. One such observational study showed a 31% risk reduction in LGA for overweight women who reduced their BMI >1 kg/m^2 prior to their second pregnancy (Ziauddeen et al., 2019). Others have explored the effects of healthy weight gain during pregnancy. Excessive gestational weight gain is associated with increased risks of LGA in all types of diabetes (Morrens et al., 2016, Bianchi et al., 2018) and in the wider general obstetric population (Siega-Riz et al., 2009). The studies exploring this have wide variations in population, methodology and weight gain descriptors but as a general message, high gestational weight gain can be associated with a 2-3 fold increase in the risk of LGA birthweight infants (Siega-Riz et al., 2009). Accordingly, the Institute of Medicine (IOM) have produced guidelines detailing recommended weight gain for women during pregnancy stratified to their pre-pregnancy BMI. These guidelines where initially developed from observational data recognising that poor gestational weight gain in mothers was associated with higher risks of fetal growth restriction and stillbirth, but have been adopted more widely in the acknowledgement that excessive weight gain can have deleterious effects on pregnancy also (Institute of Medicine, 2009). The IOM guidelines for gestational weight gain are shown in table 1.3. Observational data from a Canadian population with predominantly healthy pre-pregnancy BMI (62%) have shown that women who exceed the IOM weight gain recommendations in the third trimester have >2-fold risk of LGA, but importantly that those who exceed the recommended weight gain in the second trimester but subsequently lower weight to within recommendations in the third trimester halve that risk (Bouvier et al., 2019).

Obesity intervention is challenging for public health teams to implement given the complex interaction between medical, genetic, social and local infrastructure factors that are needed for success in obesity programmes. There is the added complication that almost half of pregnancies are unplanned (Health Matters: Reproductive Health and Pregnancy Planning (Public Health England), 2018). Lifestyle intervention in the form of combined antepartum and postpartum counselling sessions alone have proven ineffective at reducing

excessive gestational weight gain, even in a seemingly motivated trial population with approximately 90% participation in 4 sessions across all trimesters (Kunath et al., 2019). Meta-analysis of dietary intake and physical activity has shown that dietary interventions, particularly those involving calorie counting have the largest effects on gestational weight reduction in the overweight population. However, the optimal implementation in terms of recommended calories and macronutrients is less clear. Interventions in the RCTs studied included calorific intake ranging from 1200-2060 kcal/day (Shieh et al., 2018). In the same metanalysis, physical activity alone did not have significant effects on weight loss.

Table 1.3: Institute of Medicine Guidelines for recommended healthy gestational weight gain according to maternal BMI entering pregnancy

Pre-pregnancy BMI category	Recommended gestational weight gain
Underweight (BMI <18.5 kg/m^2)	12.5-18 kg
Healthy weight (18.5-24.9 kg/m^2)	11.5-16 kg
Overweight (25.0-29.9 kg/m^2)	7-11.5 kg
Obese ($\geq$30.0 kg/m^2)	5-9 kg

Adapted from Institute of Medicine 2009 guidelines (Institute of Medicine, 2009)

1.1.4.2 Neonatal hypoglycaemia

Normal physiology demonstrates that at birth, the healthy infant has blood glucose levels that are approximately 70% of maternal blood glucose, but rapidly falls to levels around 3.1 mmol/L one hour post birth, rising to 3.8 mmol/L by 4 hours postnatally and further increases to >4 mmol/L by 72 hours of age (Srinivasan et al., 1986). Fetal hyperinsulinaemia occurs in utero in response to maternal hyperglycaemia (Pedersen, 1952). In diabetes, when an infant is removed from the hyperglycaemic intrauterine environment, they are at risk of

hypoglycaemia as the hyperinsulinaemia persists transiently in the absence of high glucose substrate and exaggerates this normal physiology. The most widely accepted definition of neonatal hypoglycaemia is blood glucose <2.6 mmol/L but there is debate internationally with some societies recommending lower and higher treatment thresholds, depending on age of the infant and need for treatment above normal infant feeding regimes. Controversy exists due to a paucity of high quality evidence on hypoglycaemic thresholds and their treatment on developmental outcomes, particularly in the asymptomatic infant (Dani and Corsini, 2020). Using the 2.6 mmol/L diagnostic threshold, up to 40% of infants born to mothers with diabetes (PGDM, DIP and GDM) will experience hypoglycaemia postnatally (Simmons et al., 2000, Yamamoto et al., 2019, Voormolen et al., 2018). Important risk factors for neonatal hypoglycaemia include higher maternal glycaemia in the second and third trimesters of pregnancy (Yamamoto et al., 2019), higher maternal glycaemia at diagnosis of GDM indicating a more severe diabetes phenotype (Simmons et al., 2000), and LGA birthweight which increases risk 2-3 fold when compared with appropriate for gestational age birthweight infants (Voormolen et al., 2018, Yamamoto et al., 2019).

Neonatal hypoglycaemia is not only life threatening at the time, but has additional implications for healthcare resource (increased need for neonatal specialist care admissions) and there are concerns of adverse effects on longer term neurological sequelae for offspring (Shah et al., 2019). A systematic review showed that neonatal hypoglycaemia was associated with a 3-fold risk of visual-motor impairment, 2.5-fold risk of impaired executive function in early childhood, >3-fold risk of neurodevelopmental problems and 2-fold risk of low literacy and numeracy attainment (Shah et al., 2019). Studies in this review included hypoglycaemia ranges from less than 1.1-2.6 mmol/L, and quality of evidence was deemed low highlighting a need for ongoing work here to better define acceptable lower limits of glycaemia in neonates. Furthermore, studies included multiple risk factors for hypoglycaemia and were not diabetes specific. Two diabetes specific cohorts within this metanalysis showed conflicting results, with one group showing reduced motor, attention and perception functions at

age 8 years following neonatal hypoglycaemia and the other showing no difference in development by age 4.5 years (Stenninger et al., 1998, Haworth et al., 1976). A larger more recent study has confirmed an association with neonatal hypoglycaemia and impaired executive and visual motor function, and in particular is associated with severe or recurrent hypoglycaemia. Severe and recurrent hypoglycaemia was found more commonly in infants born to mothers with diabetes than those without (14% versus 6%) (Mckinlay et al., 2017).

1.1.4.3 Congenital anomaly

In pregestational diabetes, rates of congenital anomaly are significantly higher than that seen in the background obstetric population (2 to 10-fold higher) (CEMACH, 2005, Hawthorne et al., 1997, Casson et al., 1997). The aetiology of this is related to direct toxic effects of hyperglycaemia on embryogenesis. There is a linear association with higher maternal HbA1c in early and immediate pre-pregnancy stage and the risk of congenital anomaly (Guerin et al., 2007, Ludvigsson et al., 2018). Metanalysis has shown that those mothers with pregestational diabetes and peri-conceptual HbA1c of 5.5% (37 mmol/L) have a 2% absolute risk of congenital anomaly in their offspring, but this rises massively to 20% risk if HbA1c > 13.9% (128 mmol/L) (Guerin et al., 2007, Kitzmiller et al., 1991). The most common major anomalies seen in pregnancy complicated by diabetes are cardiac malformations, neurological (including neural tube defects) and spinal abnormalities, urogenital and gastrointestinal tract abnormalities, and musculoskeletal anomalies (Guerin et al., 2007). Folic acid supplementation (400 µg per day) has been shown in the general obstetric population to reduce incidence of neural tube defects by up to 70%, with an RCT showing high dose (4 mg per day) to have beneficial effects on prevention in high risk non-diabetic pregnancies (1991). Diabetes would be considered a high-risk pregnancy. Taken together, good glycaemic control and high dose folic acid supplementation is therefore recommended in the preconceptual and first trimester period to minimise the risk of congenital anomaly.

Some of the medications used for vascular risk management in diabetes in the non-pregnant population may have a teratogenic effect. Examples of these include Angiotensin Converting Enzyme (ACE) Inhibitors which have been linked with increased risk of cardiac and neurological anomalies when used in pregnancy and statins where increased risk of anomaly is more debated (Cooper et al., 2006, Godfrey et al., 2012). Preconceptual preparation for pregnancy should ensure discontinuation of such medicines to minimise risk of harm to the developing fetus.

1.1.4.4 Pre-eclampsia

The risk of diabetes complicating pregnancy discussed so far have largely been determined by direct toxicity or direct physiological effects of glucose on the fetus in utero. However, some of the complications of pregnancy complicated by diabetes are less understood and pathophysiology is more complex involving interactions between the maternal and fetal metabolic environments, immune and cardiovascular systems. Specifically, preeclampsia and stillbirth risk are two such conditions that are significantly increased in pregnancy complicated by diabetes with suspected intertwined metabolic and vascular pathologies (Gibbins et al., 2016). Improved understanding of potential mechanisms in these two conditions form a significant basis of this thesis.

Preeclampsia occurs in 12-20% of pregnancies complicated by T1DM, reflecting levels that are 3-5-fold higher than the background population (Jensen et al., 2004, Owens et al., 2015). In T2DM, 10-15% of pregnancies are affected (Owens et al., 2015, Groen et al., 2013). GDM also increases risk as reflected by the HAPO study (Metzger et al., 2008), and importantly, treatment of hyperglycaemia in pregnancy reduces risk by up to 60% (Landon et al., 2009, Crowther et al., 2005). Coexisting microvascular complications further augment risk. In a Danish population with T1DM, rate of preeclampsia rose to 40% for women with microalbuminuria, and up to 60% for those with frank nephropathy (Ekbom et al., 2001).

Mechanisms of disease in preeclampsia including links to glucose physiology are discussed in detail in chapter 1.2.

1.1.4.5 Stillbirth and perinatal mortality

Women with pregestational diabetes have a 2-5 fold higher rate of stillbirth than the population with diabetes (National Pregnancy in Diabetes Audit Report (NHS Digital), 2019, Feig et al., 2014). Controversy exists as to whether GDM increases perinatal mortality. Some large population-based cohorts have shown that stillbirth risk is no higher in GDM (Feig et al., 2014), whilst others have suggested an increase (Rosenstein et al., 2012). A recent UK study showed that recognition and treatment of GDM lowered stillbirth risk. Specifically, women who had risk factors for GDM but not screened showed a 50% increased risk of stillbirth compared to those with no risk factors. When this group was examined retrospectively, those with a raised fasting plasma glucose and diagnosed as GDM had a 4x lower risk of stillbirth compared to those who had raised plasma glucose but went undiagnosed during pregnancy. The GDM treated group had comparable stillbirth rates to those women without GDM (Stacey et al., 2019). The Australian Carbohydrate study also showed a trend towards reduced perinatal mortality with treatment of mild GDM (5 deaths (1%) in the untreated group versus none in the treated group p=0.06), although the study was not powered to detect mortality differences (Crowther et al., 2005).

Aetiology of stillbirth in diabetes is multifactorial. In around a third, congenital anomaly is the underlying cause, but for the remainder it can be more difficult to establish (Lauenborg et al., 2003, Wang et al., 2019). Features of placental and umbilical vasculopathy are common in these pregnancies suggesting placental vascular insufficiency is a major contributor. A small fetal post-mortem study showed that stillborn infants from mothers with diabetes had a 14-fold increase in fetal hypertrophic cardiomyopathy compared to stillborn infants to mothers without diabetes (Lynch et al., 2020). This suggests a sustained hypoxic insult in utero, and further links into the hypothesis of fetoplacental vascular insufficiency as underlying pathology.

Glycaemia is a key risk factor in stillbirth. A study published only this year, showed that third trimester HbA1c (>48 mmol/mol), highest deprivation quintile and having T2DM versus T1DM were all independently associated with perinatal mortality (odds ratio 3.06, 2.29 and 1.65 respectively) (Murphy et al., 2021).

1.1.5 Management of diabetes in pregnancy

Management of diabetes in pregnancy focuses on achieving tight glycaemic control, monitoring and management of cardiovascular risk factors, close observation of fetal growth parameters and timing delivery to reduce the complications discussed.

Ideally, women enter pregnancy having optimised metabolic factors, but we know that this is universally difficult to achieve. The National Pregnancy in Diabetes (NPID) audit showed that less than 1 in 5 mothers with T1DM and 1 in 3 mothers with T2DM achieved the preconceptual HbA1c target of <48 mmol/mol. High dose folic acid supplementation is also advised for 3 months preconceptually, and during the first trimester to reduce risk of congenital neural tube defects but again <50% of mothers with T1DM and 20% of mothers with T2DM achieved this. Taken together, only 1 in 8 women with diabetes are considered well prepared for pregnancy ((National Pregnancy in Diabetes Audit Report (NHS Digital), 2019).

Women are seen from as early in pregnancy as possible by specialist multidisciplinary teams involving diabetologists, obstetricians, diabetes specialist nurses and midwives and dieticians. They are seen far more frequently than pregnant women without diabetes, sometimes as often as weekly, to ensure timely management of the ever-changing glucose physiology in pregnancy, and allow prompt recognition of complications.

1.1.5.1 Glycaemic control

In T1DM, insulin is an essential and lifesaving treatment. In T2DM and GDM, glycaemic control can be achieved using dietary carbohydrate restriction,

metformin and/or insulin. Treatment of T2DM and GDM tends to escalate in this pattern. As discussed, there is a continuous relationship with maternal blood glucose and the various complications of diabetes. Management of hyperglycaemia therefore aims to achieve as close to normoglycaemia as possible, without increasing the burden of hypoglycaemia. For many women, this is assessed grossly by pre- and post-prandial capillary blood glucose monitoring and/or HbA1c, although the latter is not recommended in pregnancy due to altered red blood cell survival. International guidelines have slight variations on capillary blood glucose targets to achieve this, but are broadly in the region of fasting levels < 5.3-5.5 mmol/L and two-hour post-prandial levels <6.4 mmol to 7 mmol/L (Diabetes in Pregnancy (National Institute for Health and Care Excellence), 2015, Management of Diabetes (Scottish Intercollegiate Guidelines Network) 2017). A Cochrane review found that evidence comparing glycaemic targets was very limited. Their meta-analysis of three small studies found that 'loose' control defined by a fasting glucose of greater than 7 mmol/L showed evidence of increased adverse outcomes, but at levels below this it was less clear what target was optimal (Middleton et al., 2016).

In recent years, there has been a growing evidence base for use of continuous glucose monitoring (CGM) in insulin requiring diabetes. CGM studies have strengthened our knowledge base of the relationship of glycaemia and adverse outcomes. The CONCEPPT trial demonstrated that women who used CGM from early pregnancy had significantly lower rates of LGA, neonatal hypoglycaemia, lower rates of neonatal ICU (NICU) admission and a shorter hospital stay compared to women using capillary blood glucose monitoring. The numbers needed to treat to prevent each of these outcomes is remarkable at 6 for LGA and NICU admission, and 8 for neonatal hypoglycaemia (Feig et al., 2017). CGM studies have also afforded us better knowledge of the influence that glucose variability has on fetal growth. Despite achieving similar HbA1c, women who deliver LGA infants had significant differences in glucose variability across the day in each trimester (Law et al., 2015). It has since been recognised that time in range (TIR) (defined as glucose 3.5-7.8 mmol/L) is increasingly important to obstetric risk in these pregnancies. International guidelines have suggested a

target of 70% TIR (Using Diabetes Technology in Pregnancy (Diabetes Technology Network UK), 2020). This can be difficult to achieve, but data from the CONCEPPT trial have shown that an improvement of 5% TIR in the second and third trimesters significantly reduces risk of adverse neonatal outcome (Murphy, 2019, Feig et al., 2017). Following on from this landmark trial, the UK governments announced in 2020 that CGM should be made available to all mothers with T1DM in pregnancy.

1.1.5.2 Aspirin for preeclampsia prevention

Low dose Aspirin at a dose of 75-150mg is recommended in pregnant women with diabetes from 12 weeks gestation (National Institue for Health and Care Excellence QS35, 2013). This is to improve placental vascular function and prevent preeclampsia The mechanisms underpinning this are discussed in chapter 1.2.

1.1.5.3 Timing of delivery

Timing of delivery is somewhat individualised in women with diabetes, although international guidelines recommend varying timepoint for routine delivery in women with diabetes. Women are seen frequently in the third trimester to monitor glycaemia, blood pressure (BP) and fetal wellbeing. Serial fetal ultrasound is performed throughout the third trimester to monitor growth trajectory and aid decisions around obstetric intervention in those with accelerating or faltering growth, two groups of infants that are at increased risk of adverse outcome. Obstetricians will use this information to make personalised decisions around delivery and in healthy pregnancies will aim for delivery at term. However, in cases where there is concern for fetal or maternal wellbeing, pre-term delivery may be offered. In the case of pregestational diabetes, various international guidelines recommend delivery between 37-40 weeks for women with diabetes in order to prevent stillbirth and macrosomia related delivery complications (Diabetes in Pregnancy (National Institute for Health and Care Excellence), 2015, Management of Diabetes (Scottish Intercollegiate Guidelines Network) 2017, Pregestational Diabetes (American College of Obstetricians and

Gynaecologists) 2018). In the general obstetric population, stillbirth risk increases beyond 40 weeks, and vulnerable placentas such as may be seen in diabetes can be at increased risk in these later weeks (Muglu et al., 2019). Earlier intervention, particularly before 37 weeks, can however lead to increased neonatal complications such as respiratory distress syndrome (RDS) and hypoglycaemia.

Observational studies have shown that women with PGDM undergoing induction of labour at 38 weeks similar rates of shoulder dystocia and caesarean delivery but had increased rates of NICU admission, primarily for hypoglycaemia and jaundice, compared to expectant management up to 39 weeks. However, those with earlier delivery had been selected for intervention by additional risk factors of higher BMI and more likely to require insulin treatment suggesting more challenging diabetes, and confounding effects on neonatal outcomes (Brown et al., 2019). A large observational study of over 100,000 women with GDM explored risk of stillbirth at each gestational week, compared to the risk of neonatal death in the following week to better understand the balance of risk of induced delivery versus expectant management at each gestational week. The risk of induced delivery was higher than the risk of expectant management from delivery at 36 weeks and reached equivalent risk around 37 weeks. By 39 weeks, the risk of stillbirth from expectant management was higher than neonatal death with induced delivery suggesting that optimal delivery timing on a population level for these women lies between 37-39 weeks (Rosenstein et al., 2012). Of course, these observational data have several limitations in that it does not account for the confounders that could have informed obstetric decision making across the population, and does not take into account morbidity for mothers or their offspring.

A large Canadian cohort explored risk of neonatal morbidity and mortality at each gestational week in diabetes. Specifically, adverse neonatal outcome was described as any of stillbirth, neonatal death, adverse neurological outcomes in infant, birth injury, fetal or birth asphyxia and RDS. Women with T2DM, had a 1.2-1.5 adjusted risk ratio of severe neonatal adverse outcomes if iatrogenic

delivery at or before 37 weeks gestation compared to expectant management at that stage, with no increased risk seen beyond that. In T2DM, iatrogenic delivery at 36 weeks was associated with 1.5 risk ratio and no difference from 37 weeks onwards. In the GDM group, iatrogenic delivery in 36th and 37th weeks increased neonatal risk, but beyond that was associated with reduced risk of severe adverse neonatal outcome (Metcalfe et al., 2020). Most of our information that guides current recommendations around timing of delivery is from observational studies that cannot account for confounders, and are drawing conclusions from a group of mothers that are undergoing obstetric intervention for a variety of reasons. It seems clear that for the majority of women with diabetes, allowing pregnancy to continue post-40 weeks seems undesirable, and observational data would support delivery sometime between 37-39 weeks. Specifically, where within that window requires further evidence and remains an unanswered question. This is evident in the slightly differing guidelines internationally. For women with diabetes requiring insulin or medication, NICE guidelines currently suggest delivery between 37+0 to 38+6 weeks gestation, whilst Scottish guidelines suggest delivery between 38 and 40 weeks (Diabetes in Pregnancy (National Institute for Health and Care Excellence), 2015, Management of Diabetes (Scottish Intercollegiate Guidelines Network), 2017). The ACOG guidelines are slightly more conservative suggesting delivery is not routinely needed before 39 weeks if glycaemic control is good (Pregestational Diabetes Mellitus (American College of Obstetricians and Gynaecologists) 2018, Gestational Diabetes Mellitus (American College of Obstetricians and Gynaecologists), 2018).

1.2 Preeclampsia

1.2.1 Preeclampsia definition and prevalence

Preeclampsia is a multi-system condition of pregnancy defined as new-onset hypertension occurring after 20 weeks gestation in the presence of proteinuria or other signs of end-organ damage. Pregnancy-induced hypertension (PIH) is new onset hypertension in the second half of pregnancy occurring in the absence of end-organ dysfunction. PIH can affect up to 10% of pregnancies in developed nations, with preeclampsia occurring in 1.4-4% (Duley, 2009, Wallis et al., 2008). Globally, preeclampsia features consistently in the top three causes of maternal morbidity and mortality, typically as a result of progression to eclamptic seizures, organ failure or HELLP (haemolysis, elevated liver enzymes and low platelets) syndrome. It is also associated with adverse fetal outcomes such as intrauterine growth restriction (IUGR), premature birth and stillbirth (Duley, 2009). A Swedish population cohort study identified a rate of 5.2 stillbirths for every 1000 births complicated by preeclampsia (Harmon et al., 2015). Placental-derived conditions such as these have also been linked with long-term metabolic and cardiovascular consequences for mother and her offspring (Burton et al., 2016).

1.2.1.1 Preeclampsia definitions

Prior to 2013, a diagnosis of preeclampsia required the presence of proteinuria and hypertension occurring at or beyond 20 weeks gestation (Tranquilli et al., 2013). However, over time it became apparent that inclusion of proteinuria as an essential criterion for preeclampsia diagnosis had significant clinical limitations. Firstly, accelerated de-novo hypertensive end-organ damage could occur in pregnancy despite absence of proteinuria, and was associated with adverse obstetric outcome. Secondly, false positive proteinuria was not uncommon, especially when measured grossly using urine dipstick testing. In recognition of this, the major obstetric societies updated their criteria for preeclampsia diagnosis. The most current definitions for hypertensive disorders

in pregnancy are shown in table 1.4 (Brown et al., 2018, Gestational Hypertension and Preeclampsia (American College of Obstetricians and Gynaecologists, 2020). The International Society for the Study of Hypertension in Pregnancy (ISSHP) and ACOG guidelines for preeclampsia diagnosis are broadly similar. There are subtle differences in biochemical cut-offs for end-organ damage assessment. The ACOG guidelines have two major differences to ISSHP in that fetal growth restriction is not included as a diagnostic parameter for preeclampsia but do include guidance to what constitutes signs of severe preeclampsia (Gestational Hypertension and Preeclampsia (American College of Obstetricians and Gynaecologists), 2020, Brown et al., 2018).

HELLP syndrome is also included on the spectrum of placental insufficiency and preeclampsia and is a marker of more severe disease. HELLP can occur in the absence of hypertension in up to 18% of cases (Sibai, 2004). HELLP is associated with maternal mortality (around 1%), significantly higher maternal morbidity from critical complications, and perinatal mortality in up to 34% of cases (Haram et al., 2009, Sibai, 2004).

Clinically, early-onset and late-onset preeclampsia are distinguished by a cut off of 34 weeks gestation. This is relevant since women with early-onset preeclampsia are more likely to give birth to a growth restricted infant, show evidence of placental vascular insufficiency and are at higher risk of multi-organ dysfunction and associated maternal and neonatal mortality (Raymond and Peterson, 2011).

Table 1.4: The International Society for the Study of Hypertension in Pregnancy (ISSHP) 2018 and American College of Obstetricians and Gynaecologists (ACOG) 2020 diagnostic criteria for hypertensive disorders in pregnancy

	ISSHP 2018 guidelines (Brown et al., 2018)	ACOG 2020 guidelines (2020)
Preeclampsia	New onset hypertension (≥140/90mmHg) after 20 weeks gestation accompanied by any of : 1. New proteinuria (urine protein:creatinine >30 mg/mmol, or 24-hour urine protein >300 mg or 2+ proteinuria on dipstick testing) 2. New maternal end-organ damage such as: - Renal insufficiency (creatinine>90 µmol/L) - Elevated liver transaminases >2x upper limit normal - Neurological abnormalities - Low platelets <150,000/dL - Disseminated intravascular coagulopathy 3. Uteroplacental dysfunction - Fetal growth restriction	New onset hypertension (≥140/90mmHg) after 20 weeks gestation on 2 occasions at least 4 hours apart, and any of: 1. Proteinuria (>300 mg in 24 hour or protein:creatinine >0.3 mg/dL or 2+ on dipstick (dipstick least preferred) 2. Maternal organ damage: - Renal impairment (creatinine >100 µmol/L or doubling of creatinine) - ↑ transaminases >70µL or >2x upper limit normal - Severe upper abdominal pain without other cause - Cerebral or visual symptoms - Low platelets <100,000/dL - Pulmonary oedema
Severe PE		BP ≥ 160/110 mmHg on two occasions 4 hours apart and/or any of the non-proteinuria clinical features above
Pregnancy induced hypertension	De novo hypertension (>140/90 mmHg) occurring after 20 weeks gestation in woman with previously normal BP without proteinuria or end-organ manifestations of PE	New onset BP ≥140/90 mmHg (2 readings 4 hours apart) occurring after 20 weeks gestation in woman with previously normal BP, and in absence of proteinuria or end-organ damage Severe if BP ≥160/110 mmHg
Chronic hypertension	Hypertension (>140/90 mmHg or on treatment) diagnosed pre-pregnancy or before 20 weeks gestation Superimposed preeclampsia can occur in these patients	Hypertension (>140/90 mmHg or on treatment) diagnosed pre-pregnancy or before 20 weeks gestation Superimposed preeclampsia can occur in these patients

1.2.2 Pathophysiology of preeclampsia

The pathophysiology is complex and not yet fully understood but ultimately is thought to be a result of impaired placental vascular development and function, and dysregulated placental perfusion. In early onset preeclampsia (<34 weeks), abnormal placental development resulting in placental ischaemia is a likely primary pathology, compared with late onset where maternal vascular insults lead to reduced placental perfusion, although overlap exists (Raymond and Peterson, 2011).

There are two distinct phases of preeclampsia development beginning with abnormal invasion of trophoblast cells into the uterus post-conception with impaired remodelling of maternal uterine spiral arteries in early pregnancy, resulting in high resistance uteroplacental circulation. This is followed by impaired placental perfusion causing release of inflammatory cytokines and angiogenic mediators into maternal circulation resulting in maternal systemic endothelial dysfunction (Burton et al., 2019).

Numerous triggers for pathogenic embryo implantation have been identified. These include an exaggerated immunological reaction between maternal and fetal tissues, a preceding impairment of maternal circulation and altered placental vascular development due to imbalanced angiogenic and growth factors. Several markers of angiogenesis, endothelial dysfunction and inflammation are disrupted in preeclampsia (Carty et al., 2008, De Vivo et al., 2008, Zeisler et al., 2016, Sheikh et al., 2016). Maternal risk factors for preeclampsia include nulliparity, older age, obesity, autoimmune disease, smoking and diabetes, all of which can contribute to disruption in these regulatory pathways (Kaaja, 2008).

The mechanism and presentation of disease is heterogeneous which hinders development of early predictive, diagnostic and therapeutic markers. The next focus of this chapter will discuss normal placental and maternal vascular development, the hormonal, angiogenic and inflammatory factors that influence

this, and put into context how these factors might be disrupted in preeclampsia and diabetes.

1.2.2.1 Normal pregnancy and placental vascular development

The placenta is a unique vascular organ that develops during pregnancy and is responsible for transport of nutrients and gas exchange from the maternal circulation to the developing fetus, and removal of waste products from the fetal circulation. It also acts as a selective barrier to protect the fetus from some harmful molecules crossing from maternal to fetal circulation, and has endocrine functions that help support the development of a healthy pregnancy.

Placental development begins with implantation of the blastocyst in early pregnancy. When the ovum is fertilised by a sperm cell in the fallopian tube, a zygote is formed. This zygote undergoes multiple cellular divisions by mitosis, and at approximately day five following fertilisation reaches a critical cell mass and is termed the blastocyst. The blastocyst is a fluid filled structure with two layers of cells, the innermost cell mass called the endoderm which will develop into fetal tissue, whilst the outermost layer contains trophoblast cells which will later become placental tissue. Blastocyst formation is regulated by various genes concerned with ion channel activation and production of growth factors (Watson, 1992).

At day 6 to 7 post-fertilisation, the trophoblast imbeds on the uterine endometrial surface and begins the process of implantation. During this, the trophoblast cells proliferate into two layers. The outer layer forms from fused trophoblast cells that form multinucleated aggregates called syncytium. Beneath the syncytium, is a layer of proliferating cytotrophoblast cells. Under the influence of progesterone, the endometrium undergoes remodelling in early pregnancy to become the decidua. During the first couple of weeks of implantation, the syncytium invades into the decidua until the blastocyst is completely embedded within it (Turco and Moffett, 2019).

The underlying cytotrophoblasts continue to proliferate through the syncytium to produce primary villi channels surrounded by an outer layer of syncytiotrophoblasts. Branching continues to create secondary villi surrounded by vacuoles known as the intervillous space. The cytotrophoblasts then invade through the syncytium and surrounds the embryo creating a shell in contact with the maternal decidua. The embryo is now surrounded by three layers – the innermost layer which is the chorionic plate, the villous layer in the middle and the cytotrophoblastic shell on the outside. This is all complete within the first three weeks following fertilisation. The innermost layers will form the fetoplacental circulation, whilst the shell is instrumental in forming the uteroplacental circulation (Turco and Moffett, 2019).

At around 8-10 weeks of gestation, extravillous cytotrophoblasts from the cytotrophoblastic shell migrate further into the maternal decidua, and importantly into the uterine spiral arteries at 14-16 weeks. Here, they replace maternal endothelium and destroy smooth muscle structure in the high resistance, low capacitance spiral arteries and turn them into low resistance, high capacitance arteries with reduced vasoreactivity. Such features are important to maximise blood flow and perfusion of the uteroplacental unit. The extravillous cytotrophoblast cells temporarily plug the spiral artery during remodelling to prevent blood flow into the intervillous space until development of functional uteroplacental and fetoplacental circulations (Turco and Moffett, 2019). This is typically around end of the first trimester. Figure 1.1 demonstrates early placental development. Following formation of this basic placental unit, the placenta continues to undergo angiogenesis through to third trimester, increasing its vascular capacity to support the pregnancy.

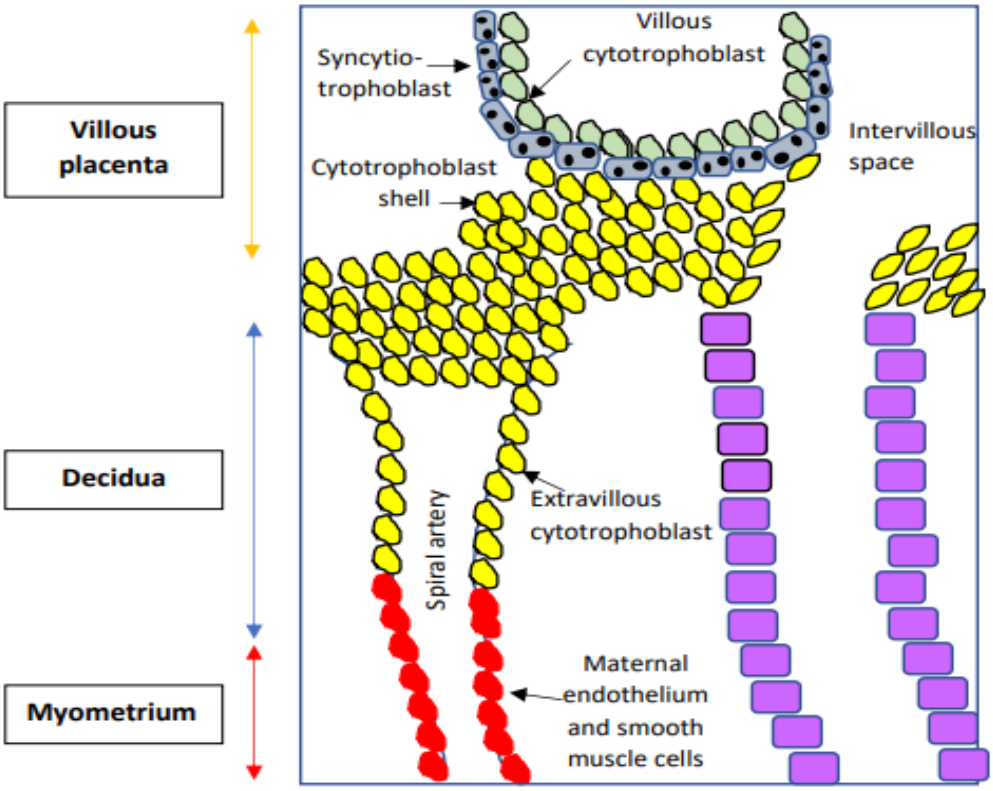


Figure 1.1: Placental development

This figure details trophoblast invasion and the different cell types involved in first trimester placental development. The inner cell mass of the trophoblast is surrounded by an inner layer of cells called the villous cytotrophoblast (green) which will form the chorionic villi and chorionic plate. This will form the fetoplacental circulation. This is surrounded by an outer membrane of multinucleated syncytiotrophoblasts (blue) which is the interface between maternal and fetal circulation across which diffusion of nutrients and gas exchange will occur and also forms a physical barrier to some harmful substances to the fetus. The outermost layer is the cytotrophoblastic cell (yellow) which invades into the maternal decidua and the spiral arteries within this. The invading extravillous cytotrophoblasts (yellow) within this replace maternal endothelium and smooth muscle cells to remodel spiral arteries in the myometrium into low resistance, high capacitance vessels to optimise blood flow from mother to fetus. This is the uteroplacental circulation. The purple cells show uterine glandular cells that can also be invaded by trophoblast cells and secrete products into the intervillous space.

1.2.2.2 Placental anatomy

The finished placental unit contains two separate circulatory systems that do not mix. The placenta is connected to the maternal circulation via the interface between the decidua and placenta, and to the fetus via the umbilical cord. The

uteroplacental circulation (maternal side) concerned with perfusion of the placenta by diffusion of oxygen and nutrients from maternal blood (via spiral arteries and intervillous space) and return of deoxygenated blood to the maternal circulation. The fetoplacental circulation's role is to allow transfer of oxygen and nutrients from placenta to the fetus (via umbilical and chorionic veins) and the removal of toxins and deoxygenated blood from fetus back to the placenta for reoxygenation (via umbilical and chorionic arteries). Figure 1.2 shows the maternal and fetal placenta macroscopic appearances.

The umbilical cord consists of two umbilical arteries and one umbilical vein bound in one cord structure by a jelly-like substance, and connects the fetal side of the placenta to the fetus. The umbilical arteries and vein form smaller branches on the fetal surface of the placenta (chorionic plate) before transcending deeper into the villous placenta. Umbilical cords in most cases insert centrally on the placenta, but in up to 10% of pregnancies can insert more peripherally (Ismail et al., 2017). This is termed marginal insertion when it inserts peripherally but still attaches within the chorionic plate, or velamentous when the cord inserts most laterally into the fibrous placental membranes (amnion) resulting in vulnerable chorionic vessels crossing between the amniotic membrane and chorionic placenta. Marginal and velamentous cord insertions are associated with higher rates of adverse pregnancy outcome relating to preterm delivery, IUGR and intrauterine death (Ismail et al., 2017). Velamentous cord insertion is the highest risk abnormal cord insertion, and affects around 1% of pregnancies (Ismail et al., 2017).

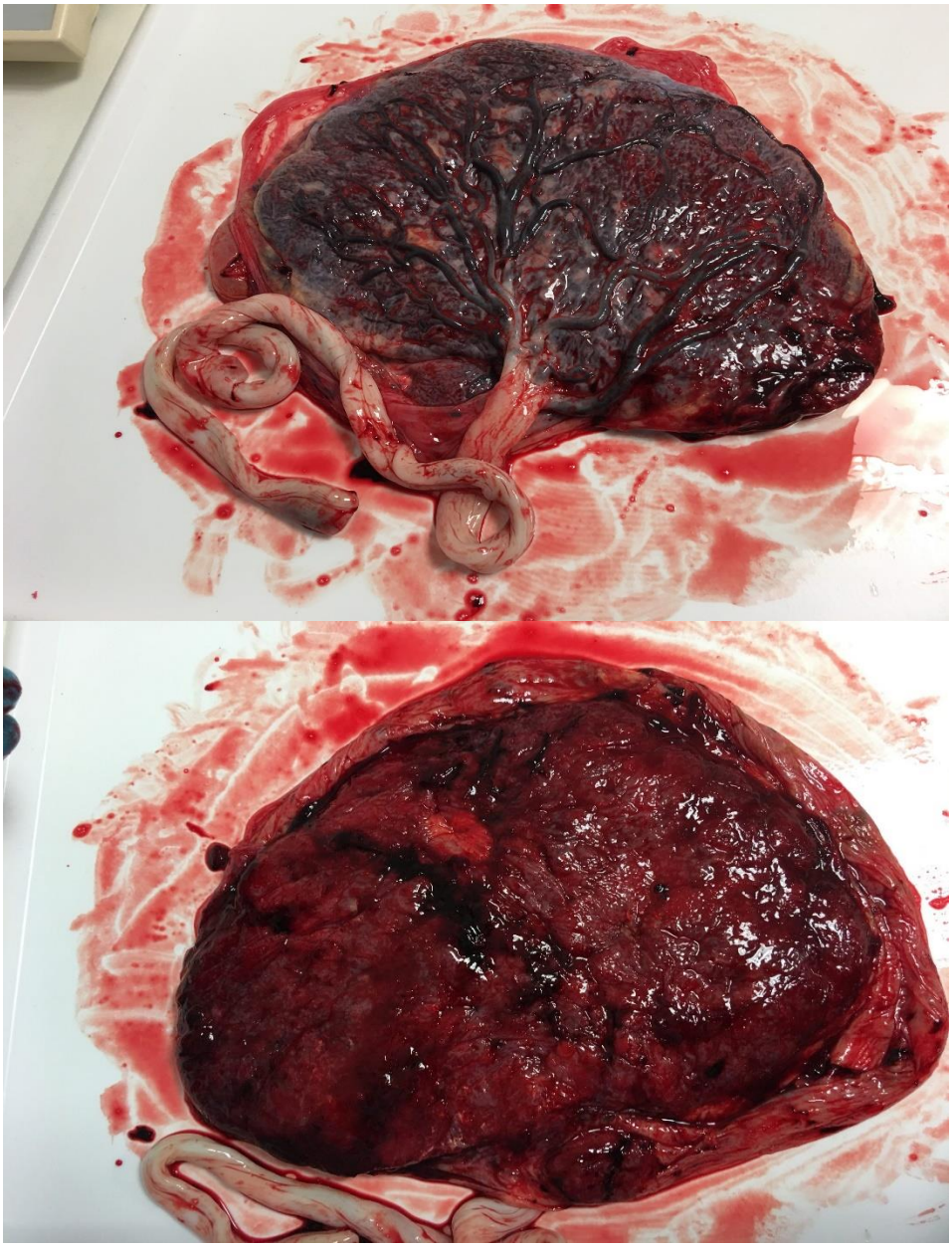


Figure 1.2: Macroscopic appearances of the placenta

The top figure shows the macroscopic appearances of the fetal side of the placenta (chorion) with marginal insertion of the umbilical cord. Branching chorionic plate vessels (arteries and veins) are visible on the chorionic plate surface and the attached white fibrous membranes of the amnion are visible. The bottom figure demonstrates the maternal side of the placenta that attaches to the uterus. The maternal side consists of multiple perfusion lobules called cotyledons that are easily visible to the naked eye. Each individual cotyledon is supplied by a spiral artery and associated anatomically with perfusion of a villous tree. In this image, an area of fibrin is noted in the upper right quadrant. Fibrin deposits are a common finding on pathological examination of placentas (up to 20%) and usually benign, but if extensive enough to cause reduced placental function can cause adverse fetal outcome (Ernst and Faye-Petersen, 2014).

Image taken by Dr Kirsty McIntyre and reproduced in this thesis with permission from her and the University of Glasgow Media Services.

1.2.2.3 Normal maternal vascular adaptation in pregnancy

At the same time as the placental vasculature is developing, the maternal systemic circulation undergoes changes from early pregnancy to optimise conditions for supporting the pregnancy. From early pregnancy, there is expansion of maternal circulating volume and cardiac output increases by 50% compared to the pre-pregnant state. This occurs in association with an almost 30% increase in maternal heart rate throughout pregnancy, and a 20% increase in stroke volume (Mabie et al., 1994, Sibai and Frangieh, 1995). The majority of these physiological adaptions occur in the first half of pregnancy, but continual changes take place into late third trimester. In parallel to these changes, the blood flow to the uterine circulation is maximised through local vasodilation in the uteroplacental unit, and to a lesser extent through systemic vasodilation in the maternal circulation. During pregnancy, up to 25% of cardiac output is directed to the uterine circulation (Mandalà, 2020). This leads to a reduced peripheral vascular resistance and lowering of BP with advancing gestation. These systemic changes occur under neurohumoral control (Goulopoulou, 2017). The effects of key pathways and hormones are discussed below.

1.2.3 Endothelial function in pregnancy

The endothelium is a monolayer of specialised cells located on the innermost surface of blood vessels. It is supported by the internal elastic lamina and together they form the tunica intima. Underneath the tunica intima lies the tunica media, which consists largely of vascular smooth muscle cells (VSMCs) with some intertwined elastic fibres. The outermost layer of the blood vessel is the tunica adventitia which is a network of collagenous connective tissue supporting the vessel's structure. Figure 1.3 shows arterial anatomy. Endothelial cells are a key regulator of vascular homeostasis. In response to physical and metabolic stimuli, they produce vasoactive substances that control vascular tone, platelet adhesion, angiogenesis and cellular inflammation.

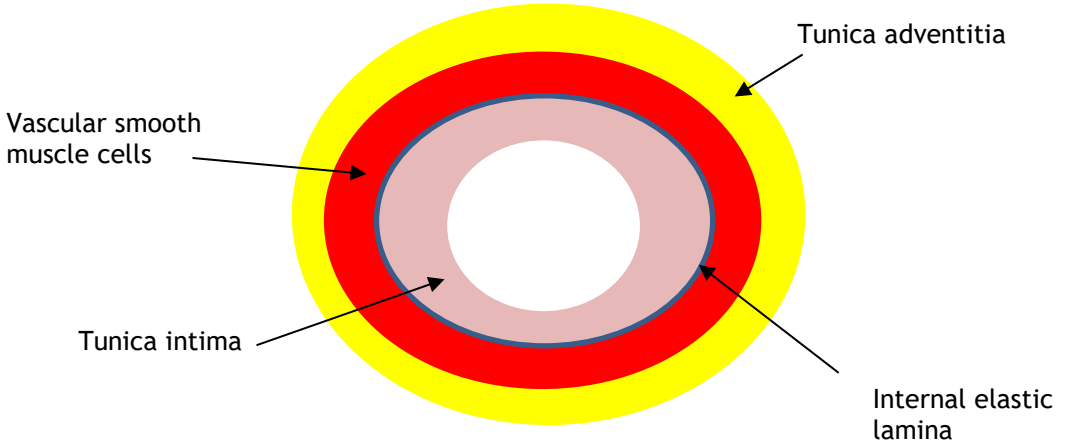


Figure 1.3: Anatomy of an artery

The innermost tunica intima layer consists of a single layer of endothelial cells, shown in pink, with a thin layer of elastic tissue (blue). Underneath this lies the tunica media layer which consists mostly of VSMCs. The outermost adventitia consists of collagen and fibroblasts that provide structural support to the vessel, as well as other important cell lines such as dendritic and progenitor cells that are involved with angiogenesis and immune function.

1.2.3.1 Nitric Oxide

Nitric oxide (NO) is a compound produced by endothelial cells from the oxidisation of L-arginine by the enzyme endothelial nitric oxide synthase (eNOS) coupled with its cofactor tetrahydrobiopterin. NO production is stimulated by mechanical force exerted on endothelium by blood flow (shear stress), or by binding of ligands to membrane receptors that stimulate intracellular calcium release and thus eNOS activation. Such ligands include but are not limited to acetylcholine, bradykinin, histamine and calcitonin gene related peptide (CGRP) (Förstermann and Münzel, 2006, Tousoulis et al., 2012).

Once produced, NO diffuses across into VSMCs to activate cyclic guanosine monophosphate (cGMP) pathways, which in turn lower intracellular calcium concentrations and promote vasorelaxation. cGMP achieves vasorelaxation

through a variety of mechanisms. Firstly, it activates protein kinase G (PKG) which stimulates cellular efflux of potassium through calcium-gated potassium channels (K_{Ca}) and inhibition of calcium release from endoplasmic reticulum within the cell. This leads to VSMC repolarisation and vasorelaxation. It also causes dephosphorylation of the myosin light chain fibres in VSMC, preventing the interaction between the smooth muscle actin and myosin fibres that are required to maintain vascular contraction (Tousoulis et al., 2012). Figure 1.4 demonstrates some of the key endothelial and VSMC pathways involved in vasodilatation.

NO activity is significantly reduced in the presence of reactive oxygen species (ROS), such as superoxide (O_2^-). O_2^- forms when eNOS becomes uncoupled, for example as a result of reduced tetrahydrobiopterin or L-arginine substrates. When formed, O_2^- and NO form the powerful oxidant peroxynitrite ($ONOO^-$) which causes oxidisation and nitration damage to cells (Alp and Channon, 2004). O_2^- can be catalysed by the enzyme superoxide dismutase (SOD) to form O_2 and hydrogen peroxide to prevent against the toxic effects of ROS generation. Healthy vascular homeostasis requires a balance of ROS production and such defence mechanisms, with imbalance leading to oxidative stress and vascular disease (Sena et al., 2018).

Experiments using nitrite and nitrate concentrations as a measure of NO activity have shown that NO concentrations in maternal serum increase from early pregnancy through to the late 3rd trimester, with levels up to 10-fold higher than that seen in non-pregnant women (Choi et al., 2002, Owusu Darkwa et al., 2018). This is largely mediated by endothelial stimulation from increasing concentrations of pregnancy hormones such as oestradiol (Sudhir et al., 1996). In the fetoplacental vasculature, laminar flow through the vessel (shear stress) induces vasodilation, an effect which is reduced if eNOS activity is inhibited (Learmont and Poston, 1996). Immunohistochemistry studies from term pregnancies have localised eNOS expression within cytotrophoblast and syncytiotrophoblasts of the placental (Schiessl et al., 2005), and NO metabolites are found in umbilical venous blood (Lyall et al., 1995). Taken

together, this suggests that NO expression is important for both systemic and placental vascular homeostasis in pregnancy.

1.2.3.1.1 Nitric oxide in preeclampsia and IUGR

In preeclamptic pregnancies, flow-mediated dilatation (FMD) of the brachial artery, a non-invasive method of assessing endothelial NO release, is attenuated compared with healthy pregnant controls. This marker of endothelial function is reduced prior to, during and after a diagnosis of preeclampsia has been made (Weissgerber et al., 2016). Higher levels of the endogenous eNOS inhibitor, asymmetric dimethylarginine (ADMA) have been found to be increased in women with abnormal uterine artery Doppler studies who later develop preeclampsia (Savvidou et al., 2003). This suggests that women who develop preeclampsia have alterations in systemic endothelial function that may contribute to deleterious effects on vasodilatation and vascular health. Disruptions in placental endothelial function in preeclampsia are more disputed. Some have shown that NO and eNOS expression is increased in the feto- and uteroplacental circulation in preeclampsia and growth restricted pregnancies as a presumed compensatory mechanism to protect fetoplacental perfusion whilst others have shown that levels are decreased (Lyall et al., 1995, Lyall et al., 1996, Schiessl et al., 2005, Sánchez-Aranguren et al., 2014). It has also been shown that mothers with preeclampsia have lower L-arginine reserves (Kim et al., 2006), and some limited studies in L-arginine supplementation have shown potential to prevent preeclampsia (Dorniak-Wall et al., 2014).

1.2.3.1.2 Nitric Oxide and diabetes in pregnancy

Endothelial dysfunction in diabetes has long been implicated in the development of diabetes and its neurovascular complications. Hyperglycaemia is known to decrease NO activity and increased oxidative stress through production of reactive oxygen species (ROS). Several mechanisms contribute to this including: oxidisation of glucose to form O_2^-, enhanced lipid peroxidation promoting ROS formation and glycation of lipids and proteins to form advanced

glycation end (AGE) products. AGE products subsequently bind to receptors on the endothelial cell (RAGE) and have a number of toxic effects mediated through protein kinase C. These include reduced mitochondrial NADPH activity and activating proinflammatory pathway mediators (Maritim et al., 2003). It is reasonable to propose that diabetes in pregnancy could have similar detrimental effects on vascular function in the maternal and placental circulation.

Two papers exploring differences in FMD of the brachial artery as a measure of endothelial dysfunction in women with GDM have shown conflicting results. The studies were small comparing 20 women per group, and at the time of starting my own research, only one had been published. One study showed that a single FMD taken between 25-39 weeks gestation was lower in GDM mothers compared with pregnant controls, but the FMD measures were not matched for gestational age (De Resende Guimarães et al., 2014). The second study demonstrated no difference between GDM mothers and controls for serial FMD measurements across first, second and third trimesters (Garg et al., 2017).

In the fetoplacental vasculature, nitrite concentrations have been shown to be significantly higher in GDM than non-GDM pregnancies suggesting increased NO production in these vessels (Figueroa et al., 2000). eNOS in human umbilical vein endothelial cells (HUVECs) is also upregulated in GDM (Di Fulvio et al., 2014). However, the NOS isoform, inducible NOS (iNOS) is also upregulated in GDM placental tissue which is pathological. iNOS is produced by endothelial cells in response to inflammatory and toxic insults, and whilst it acts to stimulate large amounts of NO production, it simultaneously promotes ROS generation (Lind et al., 2017).

Work by Sobrevia et al showed that high glucose and high insulin exposure independently increase cGMP expression and L-arginine transport in HUVECs suggesting that both insulin and glucose can stimulate NO production. However, when hyperglycaemic conditions were maintained for several hours

and insulin subsequently added, cGMP levels were significantly attenuated suggesting that endothelium-induced vasodilation is impaired by sustained hyperglycaemia (Sobrevia et al., 1996). Similar insulin resistance within HUVECS was demonstrated when they compared cGMP production from HUVEC from normal pregnancies compared with GDM pregnancies (Sobrevia et al., 1998).

1.2.3.2 Prostacyclin and thromboxane

Prostacyclin (PGI_2) is an endogenous prostaglandin with potent vasodilatory capacity. It is produced in endothelial cells by the metabolism of arachidonic acid by the cyclooxygenase (COX) pathway. Arachidonic acid is converted to prostaglandin H2 and thereafter can undergo isomerisation by prostacyclin synthase to form PGI_2, or metabolised by thromboxane synthase to form thromboxane A2 (TXA_2). PGI_2 acts to promote vasodilatation and inhibit platelet aggregation, whilst TXA_2 has the opposite actions. PGI_2 binds to G-protein coupled receptors on VSMC, activating cAMP and protein kinase A pathways. As shown in figure 1.4, this reduces intracellular calcium by preventing release from sarcoplasmic reticulum stores, inhibits MLCK activity responsible for myosin phosphorylation and activates various K channels on the cell membrane to promote ion efflux and repolarisation. These effects cause VSMC relaxation. Activation of cAMP and PKA pathways in platelets also inhibits platelet function and thrombus formation (Mitchell and Kirkby, 2019).

PGI_2 expression is significantly higher in the second and third trimesters of pregnancy compared with the non-pregnant state (Goodman et al., 1982). Whilst PGI_2 levels increase across pregnancy, there is a simultaneous decrease in TXA_2 (Wang et al., 1991). In vitro studies have shown that oestradiol stimulates PGI_2 expression in HUVECs (Sobrino et al., 2010). PGI_2 contributes beneficial vascular effects in pregnancy promoting vasodilatation and inhibiting platelet aggregation and contributes to the reduced responsiveness

to angiotensin II in pregnancy. It also promotes relaxation of uterine smooth muscle thereby quiescing uterine contractile activity.

1.2.3.2.1 Prostacyclin and thromboxane in PE

In preeclampsia, PGI_2 expression (measured by urinary metabolites) is reduced whilst TXA_2 expression is several folds higher compared with healthy pregnancy. Furthermore, these changes occur far earlier in pregnancy than when preeclampsia becomes clinically apparent, signalling that endothelial dysfunction is occurring before disease is evident. This creates an imbalance between the PGI_2 vasodilatory effects and vasoconstrictive TXA_2 in favour of a procontractile and prothrombotic phenotype (Mills et al., 1999). The cause for this imbalance is not yet fully elucidated. One contributing factor is that oxidative stress and ROS production is increased in preeclampsia which leads to increased production of lipid peroxides. Lipid peroxides promote COX activity but downstream of this inhibit prostacyclin synthase (Warso and Lands, 1983). Circulating lipid peroxides are increased in the serum of women who have preeclampsia (Madazli et al., 1999). There may also be direct or indirect hormonal influences. In preeclamptic placentas, incubation with high doses of progesterone inhibited prostacyclin production (Walsh and Coulter, 1989).

1.2.3.2.2 Prostacyclin and thromboxane in diabetes in pregnancy

Similar disruptions to prostacyclin and thromboxane have been demonstrated in hyperglycaemic pregnancy. Prostacyclin and COX-2 expression is downregulated in HUVECs from mothers with insulin-treated diabetes compared to healthy controls (Bolego et al., 2006). Thromboxane metabolites are also increased in the urine of mothers with T1DM (Vanassche et al., 1993). Prostacyclin metabolites have been found to be lower in the umbilical artery in GDM to a similar level seen in severe PIH (Johnstone et al., 1988). Antioxidant levels are lower in women with GDM than healthy controls and

may contribute to reduced antagonism of lipid peroxidation (Parast and Paknahad, 2017, Sharifipour et al., 2020).

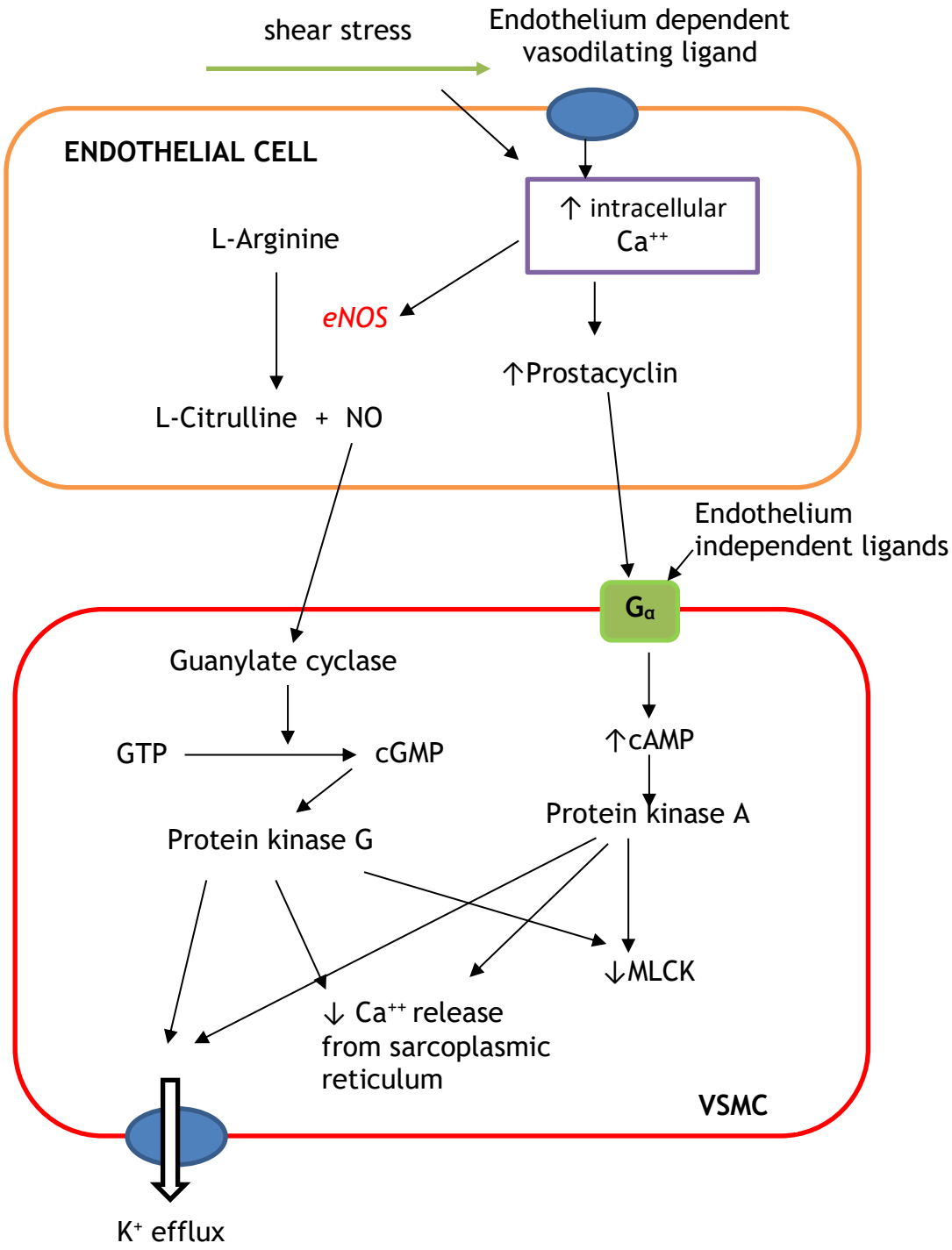


Figure 1.4: Endothelial and VSMC vasodilation pathways

In order for VSMCs to contract, they require a combination of increased intracellular calcium concentrations, cellular depolarisation and phosphorylation of myosin light chains to allow sliding of actin and myosin fibres. Inhibition of these steps within VSMCs promote

vasorelaxation and dilatation. Vasodilatation can be produced through an endothelium-dependent manner or endothelium-independent.

NO is one of the main endothelium dependent vasodilators. NO is produced in the endothelial cell from the oxidisation of L-arginine by endothelial NO synthase (eNOS). eNOS is activated in the presence of raised intracellular calcium stores and by phosphorylation from tyrosine kinase pathways. eNOS can be activated by "shear stress" (mechanical force from blood flow on cell), inflammatory cytokines, or by ligands binding to membrane receptors that activate the calcium and tyrosine kinase pathways. Once produced, NO diffused across the endothelial cell wall into the neighbouring VSMC where it activates soluble guanylate cyclase (GC) to increase cGMP pathways. cGMP activates protein kinase G which has several mechanisms: 1) Activates calcium gated K^+ channels promoting K^+ efflux and cellular repolarisation 2) inhibition of Ca^{++} release from the sarcoplasmic reticulum thereby reducing intracellular calcium stores 3) inhibits the enzyme myosin light chain kinase (MLCK) which is responsible for phosphorylation of myosin light chains. Together, these actions promote vasorelaxation.

Prostacyclin as another vasodilator produced within endothelial cells from the breakdown of arachidonic acid by the cyclooxygenase (COX) enzymes. Prostacyclin binds to G-protein (Gα) receptors on the VSMC that then activate cyclic AMP (cAMP). cAMP in turn activates protein kinase A which acts similar to protein kinase G by 1) activating K^+ channels to promote ion efflux and repolarisation 2) decreasing release of Ca^{++} from intracellular organelles 3) inhibiting MLCK activity.

Independently of endothelial production of NO or prostacyclin, ligands can bind to receptors on the VSMC wall to activate cAMP and cGMP pathways.

1.2.3.3 Endothelin

Endothelin-1 (ET-1) is a peptide produced by endothelial cells that promotes vasoconstriction, cellular proliferation and inhibits cellular death. It is produced from enzymatic cleavage of the inactive precursor, big-ET1. ET-1 expression is upregulated by low shear stress, hypoxia, adrenaline, angiotensin II, thrombin, free radicals and inflammatory cytokines, whilst NO, cGMP and PGI_2 inhibit its release (Kowalczyk et al., 2015). ET-1 binds to G-protein coupled receptors on endothelial and VSMC membranes. There are two subtypes of receptors – type A (ETA) and type B (ETB).

When ET-1 binds to ETA on the VSMC, it acts to promote calcium influx through ion channels and promotes phospholipase C pathways which stimulate calcium release from the sarcoplasmic reticulum. The net effect is of a rise in intracellular calcium concentrations promoting contraction. ET-1 also activates protein kinase C and mitogen activated protein kinase (MAPK) to promote calcium-independent contraction and cellular proliferation (Kowalczyk et al., 2015). ETB receptors are found on endothelial cells (ETB_1) and VSMC (ETB_2). When bound to the ETB_1 receptor, NO release is stimulated.

When ETB_2 is bound, it has similar procontractile effects on the VSMC cell as described for ETA but simultaneously promotes PGI_2 expression (Kowalczyk et al., 2015).

ET-1 and ETB receptors have been localised to several cell lines in the placenta – the endothelial cells, the syncytiotrophoblasts and cytotrophoblasts and does not appear to alter with gestational age (Wilkes et al., 1993, Barros et al., 2001). This is demonstrated in other studies that show no significant trend in plasma ET-1 levels across healthy pregnancy (Wolff et al., 1997). In normal pregnancy, ET-1 and Big-ET1 levels are reduced compared with non-pregnant controls (Sudo et al., 1993).

1.2.3.3.1 Endothelin-1 in preeclampsia

Circulating ET-1 and Big-ET1 levels are higher in women who have preeclampsia compared to healthy controls (Sudo et al., 1993, Aggarwal et al., 2012, Taylor et al., 1990). Numerous triggers can account for this. In preeclampsia pregnancies, the ratio of Big-ET1:ET1 is reduced suggesting that there is reduced converting enzyme (Sudo et al., 1993). Also, one of the key features of preeclampsia is the placental production of anti-angiogenic factors, inflammatory cytokines and hypoxia inducible factors (HIF) prior to development of preeclampsia These subsequently stimulate ET-1 expression. As such, there has been interest in whether antagonism of ET1 receptors might be of therapeutic benefit in preeclampsia but animal models have shown deleterious effects on fetal survival (George and Granger, 2011).

1.2.3.3.2 Endothelin-1 in diabetes in pregnancy

There is a paucity of studies exploring ET-1 and receptor expression in pregnancy complicated by diabetes and results are conflicting. ET-1 levels have been shown to be elevated in the serum of women with insulin-dependent diabetes across all stages of pregnancy (Wolff et al., 1997). Insulin is also a known stimulator of ET-1 expression. However, as Wolff et al explain, insulin secretion is higher in normal pregnancy than in the non-

pregnant state, yet ET-1 levels are reduced. Another study showed no difference in ET-1 levels in T1DM nor GDM, but importantly these women had very well controlled diabetes as reflected by HbA1c (Swiderski et al., 2010). There was a moderate correlation with HbA1c and ET-1 suggesting that hyperglycaemic pregnancy may influence ET-1 production (Swiderski et al., 2010). There is evidence of ET-1 activation in diabetes in non-pregnant subjects so it makes sense that similar changes can occur in pregnancy. Subcutaneous arteries from women with T1DM show reduced contractility to ET-1 (Ang et al., 2001), an effect which has been hypothesised to result from reduced ET-1 receptor expression. To my knowledge there are no studies investigating ET receptor expression in the same vascular bed, but in placental tissue, ET receptor expression is reduced in GDM which may reflect a protective mechanism from ET-1 overexpression (Dieber-Rotheneder et al., 2012).

1.2.4 Hormonal influences on vascular and glycaemic adaptation in pregnancy

This next section describes some of the important hormonal alterations that occur in pregnancy and their effects on glucose physiology and vascular function in pregnancy.

1.2.4.1 human chorionotropic gonadotrophin (hCG)

hCG is a peptide hormone produced by the trophoblast cells and peaks at the end of the first trimester of pregnancy. One of its main roles is to promote progesterone production from the corpus luteum which subsequently primes the endometrium for implantation (decidualisation). hCG has a critical role in placental development as it stimulates further cytotrophoblast invasion (Lee et al., 2013), and aids cytotrophoblast differentiation into syncytiotrophoblasts. In vitro experiments have also shown that hCG promotes angiogenesis, HUVEC and VSMC proliferation (Berndt et al., 2009), an effect which is at least partially due to altered expression of proangiogenic factors such as vascular endothelial growth factor (VEGF) (Brouillet et al.,

2012). The role of VEGF in placental development is discussed in section 1.2.5. In non-pregnant women, hCG administration decreased uterine artery resistance suggesting vasodilatory capabilities that could be beneficial in optimising uterine blood flow in the early stages of pregnancy. These effects were accompanied by an increased expression of the endothelial derived vasodilator(Toth et al., 1994).

1.2.4.2 Oestradiol

In the early stages of pregnancy, oestrogens are produced from the corpus luteum but as the placenta develops, it takes over as the main source of oestrogen. There are 4 different oestrogens produced: oestrone (E1), oestradiol (E2), oestriol (E3) and oestetrol (E4). Of these, E2 is the most abundant with levels increasing with advancing gestation to reach concentrations up to 100 times higher than seen in the non-pregnant state. Interestingly, the placenta lacks the enzymatic activity required to convert progesterones to androgen precursors, and so is reliant on maternal and fetal adrenal glands as a source of dehydroepiandrosterone (DHEA). This DHEA can then be converted to testosterone and subsequently aromatised by the syncytiotrophoblasts (Costa, 2016). The steroid pathways are shown in figure 1.5, with the lack of placental enzymes highlighted.

E2, and other oestrogens, act on oestrogen receptors present in the placenta, uterus and uterine artery endothelial cells and VSMCs. Oestrogens are important stimulators of endometrial proliferation, but they also have important effects on vascular and glycaemic physiology in pregnancy. E2 enhances vasodilatory capacity of arteries through upregulation cAMP pathways and increased production of eNOS (Storment et al., 2000, Napso et al., 2018). It also promotes angiogenesis in rodent uterine arteries and in HUVEC cultures (Morales et al., 1995). Taken together, oestrogens have important roles in endometrial priming for pregnancy, but also help regulate uterine blood flow in pregnancy.

Oestrogen has important effects on glucose physiology in pregnancy also. In animal models where oestrogen effects are blocked, there is notably decreased insulin sensitivity and increased adiposity. Conversely, high oestrogen environments lead to improved insulin sensitivity, reduced hepatic gluconeogenesis and protective effects from oxidative stress and preservation of ß-cell mass (Napso et al., 2018, Tiano and Mauvais-Jarvis, 2012). Several studies have found lower oestradiol concentrations in pregnancy complicated by diabetes (PGDM and GDM) (Montelongo et al., 1992, Qi et al., 2017) and preeclampsia (Berkane et al., 2017, Hertig et al., 2010) suggesting impaired placental steroidogenesis.

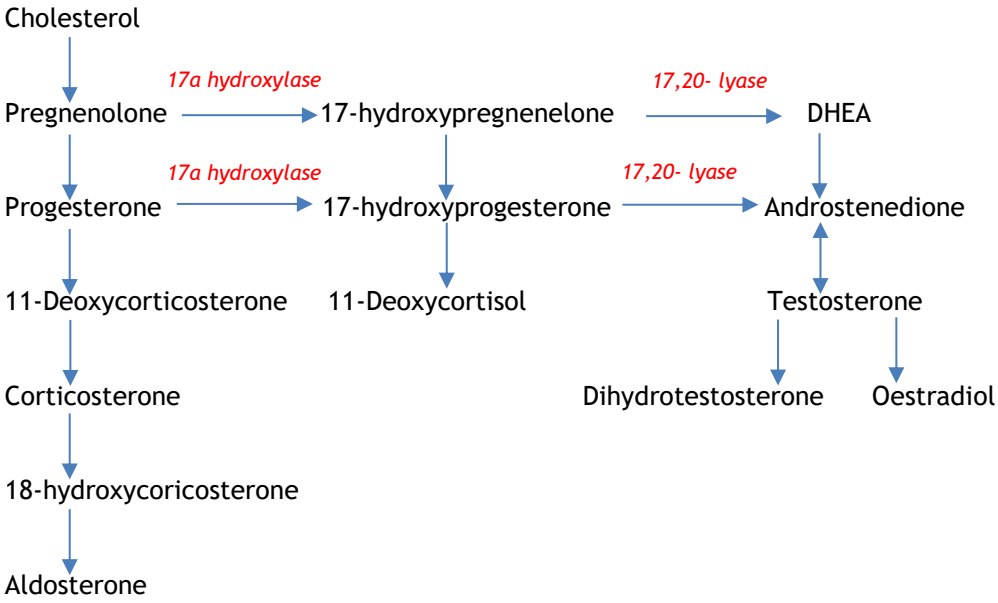


Figure 1.5: Steroid hormone pathway with highlighted enzymes that are lacking in placenta

This figure demonstrates steroid hormone production from a cholesterol precursor. Several hydroxylase enzymes are involved at each stage of the steroid conversion pathway. Those enzymes highlighted in red are essential for conversion of pregnenolone and progesterone to androgen steroid hormones, but these enzymes are not produced within the placenta. Therefore, placental production of androgens and subsequent aromatisation to oestradiol requires another source of androgen hormone. DHEA androgen is produced by the maternal and fetal adrenals and can be converted to oestradiol in the placenta.

1.2.4.3 Progesterone

In the first weeks of pregnancy, the corpus luteum (temporary remnant of the ruptured ovarian follicle post-ovulation) produces high levels of progesterone under the influence of hCG. After 6-8 weeks gestation, hCG levels decline and the syncytiotrophoblasts takes over production. Progesterone levels increase with advancing gestation, reaching a plateau in late third trimester. One of the main roles of progesterone is to stimulate decidualisation, a key process in preparing the uterus for implantation and supporting placental development. Progesterone promotes immune tolerance of the pregnancy by inhibiting the cytotoxic effects of natural killer cells, enhancing T-helper 2 (Th2) lymphocyte activity over T-helper 1 (Th1) activity, resulting in lower production of deleterious cytokines (Raghupathy et al., 2005). In vascular studies, progesterone attenuates agonist-induced vasoconstriction and promotes vasodilatation suggesting beneficial vascular effects in the high concentrations seen in pregnancy. Mechanisms to achieve this include production of NO from endothelial cells and reducing intracellular calcium influx into VSMCs (Barbagallo et al., 2001, Pang et al., 2015). The vascular effects of oestrogens are thought to be more potent than those of progesterone. Considering the vascular and immunological effects, some researchers have hypothesised a role for progesterone supplementation in preventing hypertensive disorders of pregnancy. A Cochrane review summarising four trials that had investigated this found that preeclampsia was neither prevented by progesterone supplementation, nor was there evidence of benefit in reducing adverse outcomes in preeclampsia pregnancies (Meher and Duley, 2006).

In glucose homeostasis, high progesterone levels act to increase insulin resistance in the liver, skeletal muscle and adipose tissue via its action on GLUT receptors. It also enhances ß-cell insulin secretion (Kalkhoff, 1982). Insulin resistance increases with advancing gestation as part of normal pregnancy, and this occurs simultaneously to increased expression of placental hormones that promote insulin resistance. Progesterone is one such hormone. Women who receive supplemental progesterone in pregnancy are at increased

risk of developing GDM compared with healthy pregnant controls (Rebarber et al., 2007).

1.2.4.4 Human placental lactogen (hPL)

Human placental lactogen (hPL) is a peptide hormone secreted by the syncytiotrophoblasts and to a lesser extent extravillous cytotrophoblasts (Tarrade et al., 2001). Similar to progesterone, hPL expression increases with gestational age in normal pregnancy until reaches a plateau near term (Lindberg and Nilsson, 1973). It is a somatotropin that bears structural homology with prolactin, growth hormone (GH) and placental growth hormone (pGH). hPL is a ligand for prolactin receptors, which are widely expressed in placental and fetal tissue. They can also bind with lower affinity to growth hormone receptors. Several studies have shown that maternal hPL concentrations during pregnancy correlate positively with fetal and placental size (Small et al., 1987, Mänik et al., 2010, Houghton et al., 1984, Sørensen et al., 2000). The majority of hPL is found within the maternal circulation, where it acts alongside pGH to stimulate insulin-like growth factors. It also alters maternal carbohydrate and lipid metabolism in order to maximise fetal nutrient transfer and growth. Metabolic adaptations include increased maternal insulin resistance thereby increased glucose availability for the fetus, stimulated insulin production by maternal ß-cells and enhanced lipolysis (Brelje et al., 1993, Beck and Daughaday, 1967). Lipolysis produces free fatty acids and ketones which can then be used as energy substrates by the mother and fetus respectively (Costa, 2016).

hPL has been implicated in the pathophysiology of GDM. Circulating plasma and placental homogenised hPL concentrations have been found to be higher in pregnant women diagnosed with GDM versus those without diabetes (Henderson and Divon, 1998, Muralimanoharan et al., 2016), a finding in keeping with its metabolic effects. Since hPL is secreted by the syncytiotrophoblasts, others have been interested in whether levels are altered in conditions associated with placental dysfunction, such as

preeclampsia and IUGR. Studies have shown conflicting results with hPL levels in preeclampsia. Merviel et al showed no difference in the first half of pregnancy in preeclamptic pregnancies compared to those without preeclampsia (Merviel et al., 2001), whilst others have shown reduced levels in the third trimester compared to control and GDM pregnancies (Durković and Mandić, 2009, Letchworth and Chard, 1972). This likely reflects the heterogeneity and presence of concurrent metabolic risk factors within study groups, and the heterogeneity of preeclampsia pathophysiology itself. The metabolic effects of lactogens are well researched, but the vascular effects are yet to be elucidated. In rodent aortas, hPL enhances endothelial dependent vasodilatation and increases endothelial NOS phosphorylation and NO production through its action on GH receptors (Gonzalez et al., 2015). To my knowledge, no such experiments have been published on uterine or placental vascular beds.

1.2.4.5 Renin-angiotensin-aldosterone system (RAAS)

The renin-angiotensin-aldosterone system (RAAS) is a critical hormonal regulator of circulating plasma volume, sodium concentration and vascular tone, regardless of pregnancy status. The RAAS pathway is summarised in figure 1.6. Renin is secreted by the zona glomerulosa cells of the kidney in response to perceived reduced renal perfusion or low sodium concentration in the renal tubules. Angiotensinogen, a pro-hormone produced by the liver is cleaved by renin to form angiotensin I, which then undergoes further enzymatic cleavage by angiotensin converting enzyme (ACE) to form angiotensin II. Angiotensin II has multiple actions. In vascular endothelial cells, it activates angiotensin II type 1 receptors (AT_1R) to activate intracellular calcium pathways and promote vasoconstriction thereby increasing vascular tone and BP. It also stimulates the sympathetic nervous system leading to release of the vasopressor noradrenaline. In the kidney, renal perfusion is increased by vasoconstriction and Na^+ retention promoted through the activation of Na+/H+ channels. Furthermore, it stimulates the

adrenal glands to produce the mineralocorticoid, aldosterone. Aldosterone upregulates expression of Na+ channels and Na^+K^+ ATPase in the distal tubule and collecting ducts of the kidney to promote salt and water retention. These combined effects act to restore circulating plasma volume, sodium concentrations and increase BP. Increased RAAS activity has been a well-recognised pathology in systemic hypertension for a long time, and many of our current antihypertensive treatments have been designed to target RAAS specifically (Patel et al., 2017).

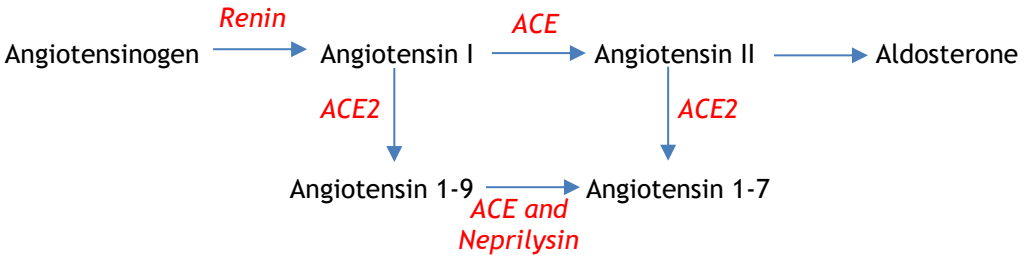


Figure 1.6: The renin angiotensin aldosterone system

This figure shows the steps involved in converting angiotensinogen to angiotensin I, angiotensin II and aldosterone. The enzymes are shown in red.

Multiple changes in the RAAS system are recognised in pregnancy, and for the most part, is upregulated in normal pregnancy in response to increasing levels of oestrogen. Renin, angiotensinogen and angiotensin II are all increased, although the rise in active renin is not seen until the second trimester (Immonen et al., 1983, Baker et al., 1990). Such changes help facilitate the plasma volume expansion required to support a healthy pregnancy. ACE2, is an enzyme structurally similar to ACE, that is found within endothelial cells and secreted by the syncytiotrophoblasts. ACE2 hydrolyses angiotensin II, a powerful vasoconstrictor to angiotensin 1-7, a vasodilating compound. Angiotensin I is also hydrolysed by ACE2 to form angiotensin 1-9, which is then converted to angiotensin 1-7 by the enzymes ACE and neprilysin. Both angiotensin II and angiotensin 1-7 are increased in pregnancy, and ACE2 may therefore play an important role in regulating uteroplacental blood flow (Pringle et al., 2011). Furthermore, others have suggested that altered expression of the angiotensin II receptors in placental and systemic circulation may occur in pregnancy (Lumbers and Pringle, 2013). Type 1 receptors (AT_1R) stimulate sympathetic nerve activity, aldosterone production and promote vasoconstriction, whereas binding to type 2 receptors (AT_2R) causes vasodilatation and opposes cellular proliferation.

Pregnancies complicated by preeclampsia have been shown to have decreased levels of serum angiotensin 1-7 with elevated levels of angiotensin II compared with normotensive pregnancies (Merrill et al., 2002). Angiotensin II has also

been found to be higher in the fetoplacental circulation of preeclampsia pregnancies and acts through binding of AT_1R in these vessels. This may contribute to a procontractile phenotype and higher resistance placental circulation that occurs in preeclampsia (Anton et al., 2008). Paradoxically, aldosterone levels are lower in preeclampsia pregnancies, and appear to be associated with lower concentration of the proangiogenic compound VEGF and higher levels of its antagonist soluble fms-like tyrosine kinase 1 (sFlt-1) (Gennari-Moser et al., 2013, Shojaati et al., 2004).

Mineralocorticoid activity in pregnancy is also mediated through non-aldosterone sources. Progesterone, which is in abundance in pregnancy, is converted to deoxycorticosterone (DOC). DOC levels are higher in serum from preeclampsia mothers versus healthy, pregnant controls (Shojaati et al., 2004).

1.2.5 Pro-angiogenic and anti-angiogenic factors in pregnancy

Normal placental vascular development is dependent on secretion of various angiogenic growth factors. A careful balance between pro-angiogenic and anti-angiogenic markers is key to optimising placental vascular development and function. VEGF and placental growth factor (PlGF) are two angiogenic factors that have a key role in pregnancy and preeclampsia development, and are two of the most widely studied.

1.2.5.1 Vascular Endothelial Growth Factor (VEGF), VEGF receptors and soluble fms-like tyrosine kinase-1 (sFlt-1)

VEGF-A is a potent vascular growth factor critical to normal placental development that has many different isoforms. It is expressed by the trophoblast (cytotrophoblast and syncytiotrophoblast) and vascular endothelial cells, and is upregulated in hypoxic environments (Chen and Zheng, 2014, Krock et al., 2011, Namiki et al., 1995). It exerts its effects primarily through binding to tyrosine kinase receptors, VEGFR1 (also known as fms-like tyrosine kinase-1 or Flt1) and

VEGFR2 (kinase insert domain receptor or KDR) found on endothelial cells (Pandey et al., 2018).

In early pregnancy, VEGF contributes to trophoblast proliferation, migration and spiral artery invasion and promotes vasculogenesis through differentiation of mesenchymal cells into endothelial precursors called haemangioblasts (Chen and Zheng, 2014). Once new vessels have formed, it has a further role in promoting angiogenesis (new vessel formation from existing endothelium) and expansion of the placental vasculature (Chen and Zheng, 2014, Ong et al., 2000). In normal pregnancy, VEGF levels are higher in earlier pregnancy than in the third trimester (Ong et al., 2000). VEGF also activates NOS pathways in endothelial cells, upregulating the production of NO and thereby inducing vasodilatation (Kroll and Waltenberger, 1998). All of these actions contribute to maximising circulatory capacity within the placenta.

sFlt1 is a splice variant of the VEGF-1 receptor, Flt-1, which when bound to VEGF acts to diminish its angiogenic effects. It is produced by trophoblasts and detectable in the maternal circulation (Clark et al., 1998). Increased expression of sFlt-1 leads to increased binding of the pro-angiogenic ligands, VEGF and placental growth factor (PlGF) resulting in lower bioavailability of active free angiogenic factors VEGF and PlGF. sFlt-1 expression increases from around week 30 gestation towards term (Palm et al., 2011).

1.2.5.2 Placental growth factor

PlGF is a pro-angiogenic factor produced most abundantly by the syncytiotrophoblast of the placenta during pregnancy, but is also expressed in lower amounts within other non-reproductive tissues such as heart, lungs, thyroid and bone. PlGF has affinity for the sFlt-1 VEGF receptor but is not bound by VEGFR2. PlGF directly promotes angiogenesis. It also has indirect effects on angiogenic pathways through binding to sFlt1, causing VEGF to have less binding capacity on sFlt1 and more to VEGFR2 which has more potent effects on tyrosine kinase pathways (Chau et al., 2017). Tyrosine kinase activation promotes angiogenesis pathways and inhibits cellular apoptosis (Ranieri et al., 2017). In a

cohort of healthy pregnant women with no signs of preeclampsia, PlGF levels increased to a peak mean concentration of 536pg/mL (139-1815pg/mL 95% CI) at 30 weeks before declining again (Saffer et al., 2013). These trends have been replicated in other studies also (Palm et al., 2011).

1.2.5.3 VEGF, sFlt-1 and PlGF in preeclampsia

Placental development and vascularisation are dependent on the balance of angiogenic and anti-angiogenic factors. Pregnancies complicated by preeclampsia, a condition associated with impaired trophoblast invasion and abnormal placental vascular development, have increased maternal circulating levels and increased placental expression of sFlt-1, with concomitant reduction in circulating levels of free VEGF and PlGF in maternal circulation (Levine et al., 2004, Maynard et al., 2003, Robinson et al., 2006). PlGF has also been shown to be lower in pregnancies complicated by severe preeclampsia compared with milder preeclampsia (Robinson et al., 2006, Levine et al., 2004). Maynard et al also showed that human umbilical vein endothelial cells (HUVECS) cultured ex vivo in serum from preeclamptic mothers show reduced angiogenesis compared with serum from healthy pregnant controls. Furthermore, those HUVECS cultured in normotensive control serum had similar attenuated angiogenic potential to the preeclampsia group when sFlt-1 added to the medium, but showed recovery of angiogenesis when VEGF and PlGF were added to the incubating solution (Maynard et al., 2003). Rodent models mimicking high sFlt-1 and low PlGF levels similar to that seen in preeclamptic pregnancy have shown numerous similar clinical features to that seen in human preeclampsia syndrome, with hypertension, growth restriction, endothelial dysfunction and glomerular endotheliosis (Maynard et al., 2003, Kumasawa et al., 2011).

As described, circulating levels of sFlt-1 (higher) and PlGF (lower) in particular, have been recognised to be altered in maternal serum at the time where the clinical syndrome of preeclampsia has been diagnosed. Levine showed that the normal increase in sFlt-1 and lower PlGF associated with advancing gestation was exaggerated in those women who subsequently developed preeclampsia in

pregnancy compared with normotensive controls. The amplified rise in sFlt-1 and subsequent decrease in PlGF began up to five weeks before clinical manifestations of preeclampsia were evident (Levine et al., 2004). Furthermore, the differences in sFlt-1 and PlGF were more marked in those individuals with severe preeclampsia or those with growth restricted pregnancies (Levine).

1.2.5.4 sFlt-1, PlGF and sFlt-1:PlGF tests in preeclampsia diagnosis

Researchers have since explored the role of incorporating sFlt-1 and PlGF testing in women presenting with suspected preeclampsia This has been borne out of a pressing clinical need to improve detection of preeclampsia since management often involves expedited delivery of the fetus, and if incorrectly diagnosed or management mistimed can lead to increased maternal and neonatal morbidity and mortality. The PROGNOSIS trial (Prediction of Short-term Outcomes in Pregnant Women with Suspected PE) showed that in women presenting with suspected preeclampsia a sFlt-1:PlGF ratio cut-off < 38 (measured on Roche Elecsys ™ immunoassay) had a high negative predictive value (NPV) of 99.3% for confirmed preeclampsia diagnosis within a week, and performed with a moderately high sensitivity and specificity (80% and 78% respectively). The converse was not true, with sFlt-1:PlGF > 38 showing poor prediction of imminent preeclampsia (positive predictive value (PPV) 37% for preeclampsia within 4 weeks) (Zeisler et al., 2016).

Another group showed that in women presenting with suspected preeclampsia prior to 34 weeks gestation, higher sFlt-1:PlGF was associated with adverse maternal and fetal outcomes in the subsequent two weeks (Rana et al., 2012). Adverse maternal outcome was defined by this group as hypertension >140/90 mmHg on two occasions plus any of: deranged transaminases, low platelets, disseminated intravascular coagulopathy, acute renal failure, placental abruption, intracranial haemorrhage, pulmonary oedema or maternal death. Neonatal adverse outcomes included expedited delivery for maternal hypertension, small for gestational age infant, abnormal umbilical artery

doppler, or fetal/neonatal death. A sFlt:PlGF ratio > 85 improved short term prediction of adverse outcomes in women presenting with suspected preeclampsia (<34 weeks gestation) when added to the classical preeclampsia signs of hypertension and proteinuria (area under curve (AUC) 0.93 combined model versus 0.84 for hypertension and proteinuria alone), and was associated with a 15-fold risk of expedited delivery within a two week period (Rana et al., 2012).

The PELICAN study explored whether PlGF (measured by Alere Triage ™ assay) could improve short term prediction of preeclampsia in women. This prospective trial included women presenting with suspected preeclampsia and investigated how serum PlGF levels taken at time of presentation related to development of preeclampsia and adverse outcome 14 days later. This study demonstrated that PlGF <5th centile for gestation before 35 weeks gestation had high sensitivity and high negative predictive value for development of preeclampsia within 14 days (0.96 [0.89-0.99] sensitivity, 0.98 [0.93-0.99] NPV) but similar to sFlt-1:PlGF studies had lower specificity (0.55 [0.48-0.61]) making the test useful for excluding imminent preeclampsia diagnosis within 14 days, but less robust in confirming preeclampsia diagnosis. The sensitivity of PlGF<5th centile was less reliable beyond 35 weeks, but exploratory analysis showed that similar sensitivity was reached if PlGF cut off <100 pg/mL was used in these later presentations (>35 and <37 weeks gestation). Interestingly, despite its low specificity for preeclampsia within 14 days, low PlGF levels had a superior AUC (0.87 [0.03]) at detecting imminent preeclampsia than other routinely used clinical parameters (BP, maternal urate levels, maternal alanine transaminase concentration, dipstick proteinuria (range 0.61-0.76 AUCs) (Chappell et al., 2013). This both highlights the lack of good predictive tests for impending preeclampsia but also highlights promising potential here for these assays in future research.

Taking these landmark trials together, there is increased recognition from learned societies that sFlt:1 and PlGF based assays can be utilised alongside clinical assessment to predict risk in women presenting with suspected

preeclampsia As an example, NICE now recommend that the Elecsys™ sFLt-1:PlGF immunoassay or Triage™ PlGF assays can be used to rule out an imminent preeclampsia diagnosis in women presenting with suspected preeclampsia between 20 and 34+6 weeks gestation (National Institute for Health and Care Excellence, 2016). Table 1.5 shows the diagnostic cut offs recommended to rule out imminent preeclampsia diagnosis. Due to lower specificity, there is concern that using PlGF assays to rule-in or diagnose imminent preeclampsia may unintentionally bias clinical decision making leading to increased unnecessary obstetric intervention and subsequent morbidity. As such, PlGF based testing is not currently recommended in UK guidelines to rule in or diagnose preeclampsia Their future potential in diagnosis is accepted though and further work encouraged to explore how their use in preeclampsia diagnosis might affect clinical outcomes (PlGF-based testing to help diagnose suspected preeclampsia (National Institute for Health and Care Excellence), 2016).

Table 1.5: PlGF and sFlt-1:PlGF assay interpretation recommendations for ruling out preeclampsia diagnosis according to NICE guidelines

Assay	Gestation	Result interpretation
Triage™ PlGF test	20+0 -34+6 weeks	≥100pg/mL rule out preeclampsia with delivery in 14 days
Elecsys™ sFlt-1:PlGF test	24+0-36+6 weeks	<38 rule out preeclampsia development within next week
Elecsys™ sFlt-1:PlGF test	20+0 weeks onwards	<33 rule out current PE

A recent UK multicentre RCT led by Professor Lucy Chappell investigated how PlGF testing incorporated into real-world clinical practice affected pregnancy outcomes. Over 1000 women with suspected preeclampsia underwent PlGF testing in this study and were randomised to either blinding of clinical teams to the PlGF results or otherwise. The concealed results group confirmed the findings of previous studies that PlGF <100 pg/mL had high sensitivity (94.9%) and high NPV (98.3%) for women developing preeclampsia needing delivery within the subsequent 14 days. The group in whom the PlGF result was known to

clinical teams, the time to diagnosis of confirmed preeclampsia was significantly lower (1.9 versus 4.1 days) with less frequent adverse maternal outcomes, but importantly was not seen to increase adverse neonatal outcomes, gestational age at delivery nor operative delivery (Duhig et al., 2019). Following on from this, the same group are currently conducting a trial to investigate whether monitoring trends in PlGF in the clinical setting can further aid clinical decision making in suspected preeclampsia and adverse pregnancy outcome (PARROT-2 trial) (ISRCTN Registry, 2019).

1.2.5.5 Angiogenic markers in other adverse outcome pregnancy

sFlt-1 and PlGF as biomarkers in adverse outcome pregnancy has the most established evidence base for preeclampsia However, low PlGF is a recognised marker of placental dysfunction and so others are interested to see if there is a role in other adverse pregnancy outcomes.

Low PlGF in the second trimester has been associated with IUGR pregnancies independent of preeclampsia diagnosis (Ghosh et al., 2013, Wallner et al., 2007). High sFlt-1:PlGF ratios and low PlGF in the second half of pregnancy are associated with IUGR (Schoofs et al., 2014, Birdir et al., 2018, Cetin et al., 2017). Low PlGF in growth restricted pregnancy is associated with higher rates of pre-term and operative delivery when compared with normal PlGF in growth restricted pregnancies (mean 31.6 weeks for very low PlGF, 34.6+2.4 weeks for low PlGF and 38.4+1.3 weeks for normal PlGF pregnancies; 83% urgent caesarean for very low PlGF group, 75% for low PlGF group, 33% for normal PlGF group) (Cetin et al., 2017). In another study of growth restricted pregnancies, a model that included low PlGF combined with other clinical risk factors and uterine artery ultrasound parameters had a modest 62% detection rate for adverse outcome (fetal death, emergency caesarean and peripartum markers of fetal distress), but importantly, despite an independent association of PlGF with adverse outcome, it did not perform better at predicting this than clinical and uterine artery imaging parameters alone (Miranda et al., 2017). This prediction

was also lower when the model was applied more widely to the general obstetric population.

Lower PlGF concentrations in the second trimester of pregnancy have also been demonstrated in pregnancies that end in stillbirth as a result of placental dysfunction (Aupont et al., 2016). The authors of this paper explored the performance of PlGF measured at 19-24 weeks gestation, in addition to maternal risk factors, fetal growth and uterine artery ultrasound doppler parameters as predictors of stillbirth. When included in a model with these parameters addition of PlGF to the model improved detection of stillbirths caused by impaired placentation to 83.6% (with a 10% false prediction rate) from 74.8%. However, PlGF did not alter the detection rate in the whole stillbirth cohort (all causes) (Aupont et al., 2016). A Cochrane review suggested that PlGF was the most accurate at predicting stillbirth, with a diagnostic odds ratio of 49.2 (Heazell et al., 2019). When applied to a hypothetical population of 1000 women with stillbirth prevalence of 1.7 per 1,000 births, low PlGF would only miss 1-2 stillbirths but would result in excess of 100 false positives with subsequent increased maternal and clinician anxiety and risk of unnecessary intervention (Heazell et al., 2019).

sFLt-1 and PlGF are altered in IUGR and stillborn pregnancies, but clinical application of these tests is currently limited. Triallists are currently planning further work to establish how these biomarkers can be used in routine clinical practice in at risk growth restricted pregnancies to guide management around obstetric surveillance and timing of delivery (Gent et al., 2020).

1.2.6 Cytokines in pregnancy

During pregnancy, the maternal immune system must adapt to develop immune tolerance to the developing fetus and prevent rejection of the pregnancy as foreign. At the same time, it must also maintain an ability to recognise and fight harmful antigens. One of the mechanisms by which it does this is to alter cytokine production in pregnancy. Cytokines are produced from CD4 T-lymphocytes, otherwise known as T-helper cells. T-helper cells can be further

divided into T-helper 1 (Th1) which produce proinflammatory cytokines that stimulate phagocytosis and promote cytotoxic lymphocyte activity, and T-helper 2 (Th2) cells that antagonise the Th-1 response, produce a combination of anti-inflammatory, IgE and eosinophilic mediated reactions and regulate antibody production. In simplistic terms, pregnancy requires a dampening of Th-1 response relative to Th-2 responses to maintain a pregnancy (Berger, 2000, Wegmann et al., 1993). As with most biological systems, homeostasis of both systems is crucial. Examples of cytokines produced by each Th subtype are shown in table 1.6, although this is not an exhaustive list. There are numerous cytokines, and discussion of them all is not in the remit of this thesis.

Table 1.6: Examples of T-helper 1 and T-helper 2 regulated cytokines

T-helper 1 cytokines	T-helper 2 cytokines
Tumour necrosis factor α (TNF-α) Interleukin 1ß (IL-1ß) Interferon-γ (IFN-γ) Interleukin-2	Interleukin 6 (IL-6) Interleukin 4 (IL-4) Interleukin 5 (IL-5) Interleukin 10 (IL-10) Eotaxin

Some researchers have explored cytokine array analysis across pregnancy and the post-partum period in normal and pathological pregnancy. In pregnancy, there is a reduction of proinflammatory cytokines from all types of Th cells (sCD40L, IL-6, IL-17, IP-10, eotaxin, and MCP-1) with a simultaneous upregulation of growth factors (Holtan et al., 2015, Shimaoka et al., 2000). Whilst both Th-1 and Th-2 responses are reduced in pregnancy, relative to each other the balance is altered in favour of the latter (Szarka et al., 2010). In pre-eclampsia, there is a shift towards a pro-inflammatory phenotype with elevated TNF-α, IL-6, IL-1ß and IFN-γ associated chemokines (Szarka et al., 2010).

1.2.6.1 Tumour Necrosis Factor α

Tumour necrosis factor α (TNF-α) is a proinflammatory Th-1 cytokine produced by macrophages, adipose tissue and in pregnancy, the placenta. It promotes apoptosis and inflammation and inhibits tumourigenesis. In pregnancy, it

promotes blastocyst implantation and uterine artery remodelling, and may have a role in preventing continued gestating of malformed fetuses, and levels are higher in pregnancy compared with the non-pregnant state (Toder et al., 2003, Staun-Ram and Shalev, 2005). In preeclampsia and IUGR due to placental vascular insufficiency, TNF-α levels are significantly higher than seen in healthy pregnancy (Azizieh and Raghupathy, 2015). TNF-α has been implicated in hypertension more widely. Mechanisms for TNFα effects include inhibition of endothelial-dependent vasodilation and upregulation of iNOS and ROS production in endothelial cells, promotion of COX vasoconstrictor pathways and atherogenesis (K and I, 2015, Zhang et al., 2009). TNF-α is also an important promoter of insulin resistance, and does so by phosphorylation of insulin signalling proteins, which then affects downstream insulin effects (Borst, 2004). Pregnancy is a state of insulin resistance, and in GDM and T2DM this is exaggerated. TNFα levels are higher in GDM pregnancies than in controls, and this is independent of BMI suggesting that enhanced adiposity is not the only contributing factor to increased levels (Xu et al., 2014, Atègbo et al., 2006). TNF-α secretion is also stimulated by a high glucose environment, further perpetuating increased secretion (Gonzalez et al., 2012). Hypoxia is another stimulant of TNF-α expression, which may be a contributing factor to preeclampsia pathophysiology.

1.2.6.2 Interleukin-6

Interleukin-6 is a widely expressed pleiotropic cytokine. Its many roles include activation of the acute inflammatory response, pyrogenic effects, inhibition of TNFα and upregulation of TNFα antagonists such as IL-10, promoting B cell antibody production, promotion of cellular proliferation (Tanaka et al., 2014). In pregnancy, it has many diverse functions also. Preconceptually, IL-6 increases post-ovulation and in response to seminal fluid and is thought to modulate the endometrial immune environment in preparation for implantation. Thereafter, IL-6 is inhibited by the pregnancy hormones oestradiol and progesterone. During placental formation, IL-6 helps regulate trophoblast invasion and promote angiogenesis. IL-6 stimulation of syncytiotrophoblasts enhances hCG and

lactogen release, hormones essential to gestational health. As pregnancy progresses, IL-6 levels increase and may contribute to the physiology of labour (Prins et al., 2012). Interestingly, IL-6 levels in pregnancy have been shown to correlate with neurodevelopment of the fetus and child (Graham et al., 2018).

IL-6 levels in maternal circulation have been found to be two-fold higher in women who have preeclampsia compared to healthy pregnant controls (Szarka et al., 2010, Mtali et al., 2019). Raised IL-6 and IL-8 concentrations have been detected in amniotic fluid significantly earlier than when preeclampsia becomes apparent (Nakabayashi et al., 1998). In the vasculature, raised IL-6 encourages endothelial dysfunction with reduction in NO activity, upregulation of ROS and AT1 receptor expression (LaMarca et al., 2011) Similarly to TNF-α, hypoxia stimulates endothelial cells to produce IL-6 (Ala et al., 1992), as does angiotensin II (Gomolak and Didion, 2014), both of which are dysregulated in preeclamptic pregnancy.

Higher IL-6 concentrations are also associated with GDM (Amirian et al., 2020). However, the effects of IL-6 on glucose homeostasis are not fully understood, and seem to differ according to source and concentration of IL-6. A review by Lehrskov et al summarises it nicely. In short, adipokine IL-6 associated with obesity alters insulin signalling that may be detrimental to hepatic insulin sensitivity, and if very high levels of IL-6 are seen will activate counterregulatory hormones to stimulate gluconeogenesis. However, short term exposure to raised IL-6, such as seen in exercise, can promote insulin sensitisation of skeletal muscles and delay gastric emptying (Lehrskov and Christensen, 2019).

1.2.6.3 Interleukin-1β

IL-1β is a potent proinflammatory cytokine. It binds to its receptor on a multitude of cells and acts to promote acute phase response proteins, upregulate COX pathways and prostaglandin production and further potentiates release of cytotoxic cytokines (TNFα) to work in synergy with its own cytotoxic mechanisms (Essayan et al., 1998). In endothelial cells, NO production is upregulated via iNOS, promotes expression of cellular adhesion molecules,

angiogenesis and is prothrombotic (Fahey and Doyle, 2019). These pharmacological effects are evident in septic shock where IL-1β is a key mediator of profound vasodilation and systemic inflammatory response.

Longitudinal assessment of IL-1β in normal pregnancy has produced conflicting results, but the larger studies support a decreasing trend in circulating levels with advancing gestation (Ferguson et al., 2014, Holtan et al., 2015). This is plausible when we consider the established roles of IL-1β seem to focus on early pregnancy development. IL-1β is thought to play a role in implantation. In IVF pregnancies, those with detectable levels had higher rates of implantation compared to those with undetectable levels (Karagouni et al., 1998). It has also been shown that IL-1β promotes decidualisation through upregulation of prostaglandin and growth factor binding synthesis. Oestradiol, which is in abundance in later pregnancy, inhibits the harmful effects of IL-1β in endothelial cells (Schaefer et al., 2005).

In preeclampsia some researchers have shown increased placental expression and circulating levels of IL-1β compared with healthy pregnant controls (Kalinderis et al., 2011, Amash et al., 2012). Conversely, others suggest a lack of evidence to support this (Deng et al., 2020).

In GDM, IL-1β expression is higher and its endogenous receptor antagonist lower than in non-GDM mothers (Vitoratos et al., 2008, Katra et al., 2016). Lower levels of the receptor antagonist were also associated with progression to T2DM post-partum (Katra et al., 2016). IL-1β has several effects in glucose physiology. Blocking of IL-1β function improves glycaemia in mice through a decrease in counterregulatory steroid production which would act to improve insulin sensitivity (Schulze et al., 2020). In animal and in vitro models, high levels of IL-1β have also been shown to promote ß-cell destruction. As such, IL-1β blockade has been a focus in recent T1DM research although advances here have been limited by negative studies in humans (Mandrup-Poulsen et al., 2010).

1.2.7 Prevention of PE

There is a major unmet need for better intervention to predict and prevent preeclampsia, but most therapeutic strategies have proven ineffective.

1.2.7.1 Aspirin

Low dose aspirin (75-150mg) is widely used clinically from 12 weeks gestation to prevent preeclampsia in women at high risk of the condition (Hypertension in Pregnancy (National Institute for Health and Care Excellence), 2010, Gestational Hypertension and Preeclampsia (American College of Obstetricians and Gynaecologists), 2020). Therapeutic mechanisms inhibit the COX pathway reducing platelet derived thromboxane production and its pro-thrombotic and vasoconstrictive effects, and improving placental perfusion. Numerous RCTs have been conducted but include different timings of prescribing in pregnancy, and doses that vary from 60-150mg. Clinical outcomes in these trials have varied. The ASPRE (Aspirin for evidence-based preeclampsia trial) is one of the largest and most recent studies. This double-blind RCT assigned high-risk women to either aspirin 150mg daily or placebo between 11-36 weeks gestation, and demonstrated that aspirin 150mg resulted in a 62% reduction in preeclampsia with secondary analysis showing a knock on effect in reduction of babies born severely preterm (<32 weeks) and therefore lower NICU stays (Wright et al., 2018, Rolnik et al., 2017). Studies using a lower 60mg dose have shown more conflicting results (Caritis et al., 1998, Sibai et al., 1993). In 2014, the US Preventative Services Taskforce assessed available evidence at the time, and their metanalysis suggested that Aspirin (60-150mg) is beneficial and should be prescribed to high risk women (as identified in table 1.7) although are less prescriptive about specific dose recommendations (Lefevre, 2014). At that time, they cited lack of evidence to show advantageous effects of any particular dose over another, nor was there evidence to support beneficial effect in timing of prescribing (pre- and post-16 weeks). Further independent meta-analysis has since been conducted, and includes the more recent RCTs. That suggests that the benefits of aspirin in preeclampsia prevention have a dose effect, where doses of 60mg and less show no benefit whilst there increasing doses (up to

150mg) correlate with lower rates of preeclampsia severe preeclampsia and fetal growth restriction. These effects are more pronounced when therapy initiated prior to 16 weeks gestation, as initiation after this was only associated with reduced incidence of preeclampsia but no impact on the other outcomes (Roberge et al., 2017).

Table 1.7: Recommendations of the US Preventative Services Taskforce for low dose aspirin in preeclampsia prevention (Lefevre, 2014)

Risk category	Definition	Recommendation
High	Any 1 of: - Previous PE - Multiple pregnancy - Pregestational diabetes - Renal disease - Autoimmune disease - Pregestational hypertension	Aspirin (60-150mg) daily recommended
Moderate	More than 1 of: - First pregnancy - BMI >30kg/m^2 - First degree relative with PE - Maternal age > 35 - Previous adverse outcome pregnancy due to placental issues - >10-year pregnancy interval - High risk ethnicity e.g., Black -	Aspirin (60-150mg) daily should be considered
Low	Previous uncomplicated pregnancy	Aspirin not recommended

1.2.7.2 Antioxidants

Since oxidative stress is a hallmark of preeclampsia pathophysiology, researchers have been interested in antioxidants as a preventative strategy. However, results from these studies have largely been disappointing. Antioxidants explored include vitamin B, vitamin C, vitamin E, Selenium and leucopene. Meta-analysis, including a Cochrane review has shown no evidence of benefit in either preeclampsia or severe preeclampsia prevention with antioxidants (Rumbold et al., 2008, Salles et al., 2012). Women who were prescribed antioxidant therapy were more likely to present with gastrointestinal side effects (Salles et al.,

2012). Lack of effect of vitamin C and E supplementation in preeclampsia prevention has also been shown in T1DM (Mccance et al., 2010).

1.2.7.3 Vitamin D and calcium supplementation

Maternal vitamin D deficiency is associated with increased preeclampsia risk (Bodnar et al., 2007). Vitamin D has several important actions in healthy pregnancy. These include regulation of fetal bone growth and development, inhibition of T-cell proliferation and promotion of a Th2>Th1 response and enhances VEGF expression and angiogenesis (Grundmann et al., 2012, Aranow, 2011). In addition, vitamin D deficiency has also been associated with higher incidence of GDM, and higher risk of fetal growth restriction (Aghajafari et al., 2013, Van Der Pligt et al., 2018). Vitamin D supplementation has been shown to 52% reduction in preeclampsia incidence on meta-analysis of 4 studies involving 499 participants. In the same metanalysis, 4 studies that included 1100 women receiving combined vitamin D and calcium supplementation showed a similar risk reduction, but importantly signalled an increase in pre-term birth (De-Regil et al., 2016). These studies universally used cholecalciferol for vitamin D supplementation, but doses varied between 400 units daily to single high-dose 25,000 unit administration, and were given at different time points in pregnancy. Most of the studies are also observational and confounding factors often accompany vitamin D deficiency, such as ethnicity, smoking status and comorbidity.

Taken together, vitamin D has numerous beneficial effects on pregnancy and may reduce preeclampsia risk. As such, sufficient vitamin D levels appear important to the health of a pregnancy. The Royal College of Obstetricians and Gynaecologists (RCOG) therefore make pragmatic recommendations for vitamin D supplementation in pregnancy. Whilst optimal dosing remains to be established, they suggest 400 units daily, doubling to 800 units daily in women who are deemed at higher risk of vitamin D deficiency (darker skin tone, lack of sunlight exposure) (Vitamin D in Pregnancy (Royal College of Obstetricians and Gynaecologists), 2014).

1.2.7.4 Low molecular weight heparin

Low molecular weight heparin (LMWH) has more recently been studied for preeclampsia prevention. Coagulopathy and thrombophilia defects are common in preeclampsia and so negating the effects of these with anticoagulation is a plausible hypothesis. Recent studies have shown that LMWH administration in pregnancy is associated with improved markers of endothelial function and pro-angiogenic markers PlGF and sFLt-1 (Mclaughlin et al., 2017). Clinical trials however have been limited by small studies and marked heterogeneity in study populations. Studies have largely focused on women who have had a past history of previous placental pregnancy complications. A Cochrane review analysis identified 7 studies reporting preeclampsia as an outcome in LMWH studies. Individual study numbers ranged from 20-224 participants, and recruited women from a variety of clinical backgrounds. These included previous preeclampsia, abnormal placental function on routine pregnancy imaging, previous placental abruption, previous intrauterine death and a diagnosis of glomerulonephritis (Dodd et al., 2013). Whilst LMWH seemed to reduce preeclampsia on combined analysis of this group, the heterogeneity makes it difficult to apply the principles to the general obstetric population. LMWH is not currently recommended for preeclampsia prevention in the UK (Hypertension in Pregnancy (National Institute for Health and Care Excellence), 2010). High quality RCTs in larger populations would help identify groups in whom LMWH may be suitable in.

1.2.7.5 Glycaemic control

While part of preeclampsia risk in pre-existing diabetes may relate to existing complications and endothelial dysfunction there also appears to be an intimate and dynamic relationship between glycaemia and preeclampsia risk. Preeclampsia risk increases linearly across the range of normal maternal glucose (Metzger et al., 2008). Third trimester maternal Hba1c in T1DM is associated with risk of preeclampsia over and above risk associated with existing diabetes complications (Holmes et al., 2011). More importantly glucose lowering attenuates preeclampsia risk. Two large treatment trials of women with mild GDM have both shown a reduction in risk of preeclampsia by 55% and 33%

respectively with glucose lowering by dietary and/or insulin intervention (Landon et al., 2009, Crowther et al., 2005). This is particularly striking as in this case a treatment started later in pregnancy (generally after 24 weeks) is reducing risk.

There has also been interest in whether specific treatments for diabetes in pregnancy might confer benefit to preeclampsia risk. Individual study results are conflicting but meta-analysis shows that metformin confers a 46% reduction in PIH, with a non-significant trend towards lowering preeclampsia risk in women with GDM compared to insulin-treated counterparts. These differences are not explained by differences in glycaemic control (Zhao et al., 2015, Barrett et al., 2014). Others have investigated whether metformin would alter placental and endothelial function using in vitro and ex vivo approaches and have shown that it does. Metformin reduces placental angiogenic factors via its actions on the mitochondrial electron transport chain (Brownfoot et al., 2016). Han et al also confirmed that in vitro metformin reduces trophoblast pro-inflammatory cytokine production in high- and low-glucose environment (Han et al., 2015). Whilst these effects of metformin appear independent to glycaemia, their effects on preeclampsia risk does not appear to confer similarly on women who do not have diabetes. Metformin in polycystic ovarian syndrome does not appear to reduce preeclampsia risk (Tan et al., 2016). Studies where women are selected for obesity also show conflicting results, with one RCT showing reduction of preeclampsia but not PIH, and no difference in another (Syngelaki et al., 2016, Chiswick et al., 2015).

1.2.8 Preeclampsia treatment

The only curative treatment for preeclampsia is delivery of the infant and placenta, thereby removing the stimulus of placentally derived factors being released into maternal circulation. However, preeclampsia can present from as early as 20 weeks and so clinical decisions around the timing of delivery need to balance the imminent risk of preeclampsia to mother and fetus, against those of preterm delivery. In term infants, delivery would be the preferred option, but

in earlier gestations, attempts to delay delivery with expectant management of BP and careful monitoring should be considered.

Clinical decisions around expedited delivery can be challenging. Some prediction scores have been developed to help clinicians stratify risk, but they focus on maternal risk only. The fullPIERS model is a risk score developed to predict maternal mortality and severe maternal adverse outcome. Using clinical parameters, it generates a percentage risk for an individual to have adverse outcome in the short term, and categorises women according to low risk (<2.5%), intermediate (2.5-30%) and high risk (>30%). Initial studies showed an AUC 0.88 in predicting adverse outcome within 48 hours (>0.7 within 7 days) (Von Dadelszen et al., 2011). Independent review has suggested that it performs well in identifying women in the high risk group who should be admitted, with less robust evidence in predicting absence of adverse risk in the low and intermediate groups (Hypertension in Pregnany NG133 (National Institute for Health and Care Excellence), 2019). It has not been validated in predicting adverse fetal outcome. Taken together, the UK guidelines suggest that such risk scores may be utilised to guide clinicians in assessment and management of preeclampsia but purposefully avoid descriptive guidance on how they should be used (Hypertension in Pregnancy (National Institute for Health and Care Excellence), 2010). Instead, they suggest considering delivery if concerning fetal or maternal features. These might include resistant BP control despite three antihypertensive agents, progressive biochemical and haematological abnormality, eclampsia, placental rupture or non-reassuring fetal assessments.

1.2.8.1 Blood pressure management

In pregnancy, guidelines define hypertension as BP >140/90 mmHg, and severe hypertension as >160/110 mmHg. There is international consensus that severe hypertension needs to be treated, but controversy about treatment of milder hypertension in pregnancy exists. UK guidelines currently suggests that both hypertensive groups should be treated, with a target BP 135/85 mmHg, aiming to keep diastolic (DBP) above 80 mmHg to prevent placental hypoperfusion

(Hypertension in Pregnancy (National Institute for Health and Care Excellence), 2010). An RCT of non-severe, non-proteinuric hypertension in pregnancy showed that treatment to a DBP target <100 mmHg (achieving a mean 90 mmHg) versus 85 mmHg (achieving target) conferred no benefit to severe maternal complications, fetal mortality or NICU admissions. However, women in the more tightly controlled group were significantly less likely to develop severe hypertension later in pregnancy (28 versus 41%) (Magee et al., 2015). A Cochrane review suggested similar with treatment of mild to moderate hypertension halving the risk of women subsequently developing severe hypertension, but with limited evidence to suggest any effect on neonatal or maternal outcomes otherwise (Abalos et al., 2018).

Pharmacotherapy options for hypertension in pregnancy include labetalol, nifedipine, methyldopa and hydralazine, with the first two being used preferentially. Magnesium sulphate intravenously is used in preeclampsia as an adjunct to antihypertensive treatments, and in the prevention and treatment of eclamptic seizures. Magnesium sulphate has several mechanisms that are beneficial in preeclampsia. In VSMCs it blocks calcium channels preventing cellular influx and promoting systemic vasodilatation, and may also enhance endothelial function (Darooghegi Mofrad et al., 2018). It alters blood brain barrier function and alters central nervous system receptors offering maternal neuroprotection against seizures, and in the preterm infant promotes brain maturation and neuroprotection (Usman et al., 2017, Euser and Cipolla, 2009).

1.3 Aims of thesis

In summary, there are increased rates of adverse outcome in pregnancy complicated by diabetes. Some of this risk is related to fetal hyperinsulinaemia and overgrowth or direct teratogenic effects of glucose on the developing fetus, but mechanisms associated with other complications related to placental function such as stillbirth and preeclampsia are less understood. Evidence from previous work indicates a linear relationship between maternal glycaemia and preeclampsia even in the later stages of pregnancy, and furthermore treatment of this glycaemia lowers this risk. One potential mechanism for increased preeclampsia and stillbirth risk is altered placental perfusion and/or function from hyperglycaemia in pregnancy. Hypertensive disorders are also more common in the pregnant population with diabetes. In the non-pregnant state systemic vascular endothelial function is impaired by a persistent hyperglycaemic environment, and it would be plausible that similar changes may occur in the systemic and placental vascular beds in hyperglycaemic pregnancy. Reduced placental perfusion or vascular dysfunction could lead to fetoplacental ischaemia and such complications in pregnancy.

The aims of thesis are therefore to:

1. Establish the prevalence of obstetric complications in the Scottish population with diabetes compared to the pregnant population without diabetes, and whether current clinical care is improving outcomes

2. Establish what demographic and clinical risk factors are associated with stillbirth in the pregnant population with diabetes

3. Determine if maternal systemic endothelial function is altered in GDM pregnancy compared to normoglycaemic pregnancy as a potential mechanism for vascular dysfunction and increased risk of hypertensive disorders, preeclampsia and stillbirth.

4. Determine if placental vascular function is altered in GDM pregnancy compared to normoglycaemic pregnancy.

2 Chapter 2: Methods

2.1 Epidemiological studies

2.1.1 Clinical databases

The epidemiological studies in this thesis were granted from, and carried out in collaboration with the Scottish Diabetes Research Network (SDRN) Epidemiology group. The SDRN is a Chief Scientist Office (CSO) commissioned group of clinical and non-clinical researchers with an interest in diabetes research. The group have roles in promotion of high quality, collaborative diabetes research in Scotland (Scottish Diabetes Research Network).

The SDRN epidemiology group, in collaboration with Information Services Division (ISD) Scotland, have produced and maintain a server with linked, anonymised data of patients with diabetes from major clinical databases in use across Scotland. The linked datasets are updated every one to two years to ensure information is up to date and undergo regular audit and scrutiny by the research teams to ensure accuracy of clinical data. Researchers can apply to the SDRN for access to anonymised linked data from the SDRN server. Prior to access, researchers must complete online learning on data handling and general data protection regulations. Suggested studies must be submitted to and approved by the SDRN and must also be approved by the appropriate ethics or Caldicott committees prior to access. For the work contained within this study, data linked from the national inpatient maternity database, Scottish Maternity Records 02 (SMR02) and the national diabetes database, SCI-Diabetes was requested. The linked databases are accessed in a pseudonymised format remotely from the server using PuTTY.exe software (https://www.chiark.greenend.org.uk/~sgtatham/putty/). Ethical approval for the work to be carried out on behalf of the SDRN Epidemiology group was granted by the Caldicott Guardians of all Health Boards in Scotland and ISD Privacy Advisory Committee.

2.1.2 Scottish Care Information (SCI) Diabetes

SCI Diabetes is the national diabetes electronic health record used across all regions of Scotland. It is used by clinicians across Scotland as part of routine clinical practice to document diabetes diagnosis, routine clinical care and outcomes of patients with diabetes. The database includes information on physician assigned diabetes diagnosis, diabetes prescription history, annual diabetes screening data (micro- and macro-vascular disease), use of diabetes technologies and diabetes education received. Patients are identified within this database using their unique patient identifier, the Community Health Index (CHI) number. CHI numbers are used routinely in medical records across all NHS systems in Scotland, therefore allowing easier linkage of records regardless of healthcare setting.

Development of a national diabetes database was first detailed in 2001 in the Scottish Diabetes Framework, as a means to promote a collaborative and improved approach to diabetes care in Scotland. Prior to this, regional diabetes databases were utilised in some health boards across Scotland. In 2004, a web-based national database was rolled out and since then has been integrated in routine clinical care for patients with diabetes across all health boards in Scotland (McKnight et al., 2008). Data from pre-2004 were added to SCI-Diabetes using a variety of sources from primary, secondary care and biochemistry records, but may not include patients who moved out of Scotland or died before this date.

Diabetes diagnosis is primarily entered into SCI Diabetes as a clinician assigned diagnosis, but the linked data undergoes further validation by SDRN algorithm utilising age at diagnosis and prescription history to improve accuracy. Specifically, the algorithm would reassign patients who had a clinician-assigned diagnosis of T1DM as T2DM if not received insulin therapy within a year of diabetes diagnosis. Similarly, would reassign as T1DM if clinician-assigned T2DM but diagnosed under the age of 30 years AND required insulin therapy within one year of diagnosis. Published work has shown that when validated against

inpatient medical records, the database includes information from over 99.5% of the Scottish population and diabetes diagnosis is validated in over 99% cases (Anwar et al., 2011). Other work validating attendance at retinal screening has suggested similar high levels of accuracy of diabetes diagnosis in SCI-Diabetes (Scottish Care Information Diabetes Collaboration).

2.1.3 Scottish Maternity Records 02 (SMR02)

The Scottish Maternity Records 02 (SMR02) database is a national database that includes clinical data relating to all obstetric inpatient admissions. The database has existed since 1975 and consists of information that is submitted from all maternity hospitals in Scotland. Comparison of delivery data with births registered on the national birth registry suggest good population coverage with >98% of all births captured (Births in Scottish Hospitals (Informations Services Division Scotland)). The SMR02 will not include those who delivered in a non-maternity hospital setting.

Data relating to maternal demographics, delivery details and neonatal characteristics are included in SMR02. Table 2.1 shows the data requested as part of this work, and accuracy of the data within SMR02 according to a 2008/09 audit of the database (Assessment of maternity data (SMR02) 2008-2009 (Information Services Division Scotland), 2010).

Other items that would have been of interest but had poor accuracy when audited included height and weight of mothers (72% and 77% respectively) and ethnicity (11% accuracy) (Assessment of maternity data (SMR02) 2008-2009 (Information Services Division Scotland), 2010). For mothers with diabetes, pre-pregnancy BMI was therefore taken from SCI-Diabetes which would have been recorded by clinicians as part of routine annual diabetes review.

Table 2.1: Accuracy of data items within SMR02 audited by ISD Scotland in 2008/09 (adapted from SMR02 (Information Services Division Scotland, 2010).

SMR02 item	% accuracy 2008/09 ISD audit
Number of deliveries this pregnancy	100
Pregnancy outcome	100
Infant sex	100
Birthweight	99
Previous pregnancies	94
Gestational age at delivery	93
Maternal smoking status during pregnancy	90
Mode of delivery	87
Maternal smoking status at booking	80

2.1.4 Diabetes and Pregnancy Outcomes in Scotland methodology

Access to data from the linked SMR02 and SCI-Diabetes was requested from years 1998 to 2013. At the time of setting up the project, 2013 was the latest excerpt of complete linked data. All information within the linked databases is visible only in a pseudonymised format. The aim of this study was to explore the prevalence of diabetes in pregnancy, associated obstetric outcomes in these pregnancies and how they compared to the obstetric population without diabetes, and the temporal trends in this data.

Initial examination of the linked data revealed a very low number of mothers with a diagnosis of diabetes that was not T1DM or T2DM (249 of 813,000 deliveries). GDM diagnosis is also grossly underestimated in SCI-Diabetes as it is not used routinely in obstetric clinics across Scotland and so this study was restricted to exploring outcomes in mothers with pregestational T1DM and T2DM only.

2.1.4.1 Study time period

The time period beginning 1st April 1998 through to 31st March 2013 was used for a number of reasons. Firstly, absolute numbers of women with diabetes delivering infants per year are modest in Scotland, and so a larger time period is required to detect meaningful differences in some less common adverse outcomes such as stillbirth. As 2013 was the latest complete set of linked data available at this time, a period of 15 years prior to this seemed a good timeframe to provide a large enough population to investigate outcomes, and to allow additional analysis for time trend. Furthermore, two previous national paper audits had been published in 1998/99 and 2002/03 and so comparison of our data against these was useful for additional validation of our data (Kernaghan et al., 2006, Penney et al., 2003).

In Scotland, fiscal years are often used to describe national statistics, as these coincide with national budget timeframes and can be used to map outcomes against health policy planning. The Scottish and UK fiscal year runs from 1st April one year to end of March the following year, and so time data in this study are presented in this format.

2.1.4.2 Study population

All women who delivered an infant, live or stillborn, at or beyond 24 weeks gestation in a Scottish maternity hospital were included. Gestational age inclusion criteria were decided based on legal definitions for stillbirth in Scotland. Deliveries that happened outside of a hospital will have been few, but these data would not have been included as an SMR02 inpatient episode and so could not be included. Figure 2.1 details the study population numbers.

2.1.4.3 Pregnancy outcome data

An analysis plan for determining pregnancy outcomes in T1DM and T2DM was outlined at the beginning of the study. Firstly, I wanted to establish the prevalence of diabetes in pregnancy in the Scottish population and the maternal demographic it affected. Then, the rates of key adverse outcomes in the

population. The pre-defined factors are listed in table 2.2. The predefined analysis was to understand the absolute rates of these in the population and whether rates were different depending on diabetes diagnosis. Secondly, to understand if rates changed across time. Following peer review for publication, examination of smoking rates and deprivation were added to the analysis.

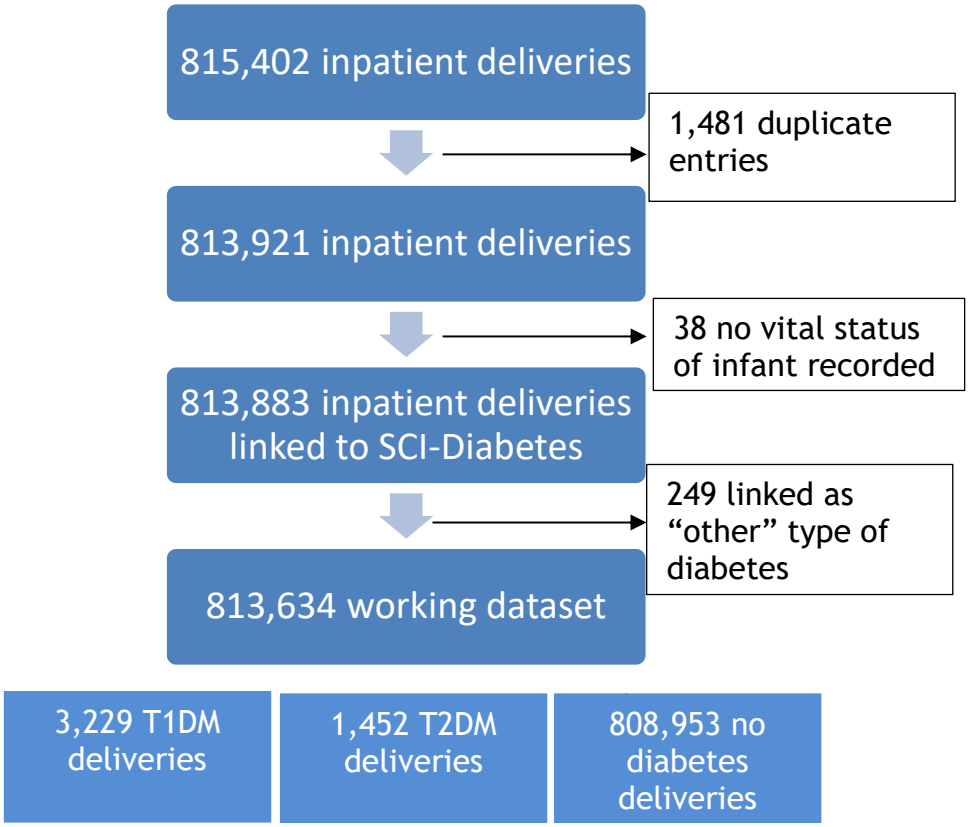


Figure 2.1 Population data included for study exploring obstetric outcomes in population with and without diabetes

This figure shows the number of records available within SMR-02 and used for the epidemiological study exploring rates of adverse obstetric outcomes in the population with diabetes compared to those without. The final number of records available for inclusion were 3229 deliveries to mothers with T1DM, 1,452 to mothers with T2DM and 808,953 deliveries to mothers without pregestational diabetes.

Table 2.2: Predefined variables of interest in defining rates of adverse outcomes in pregnancy complicated by diabetes

Maternal demographics	Infant demographics	Obstetric outcomes
Age	Singleton or multiple birth	Mode of delivery
Diabetes diagnosis	Gestational age at delivery	Rates of pre-term delivery (<37 weeks)
Duration of diabetes	Absolute birthweight	Rates of very preterm delivery (<32 weeks)
Parity	Birthweight LMS Z-score	
SIMD deprivation score	Vital status at birth	Perinatal mortality

Definitions used to define these parameters are listed below:

Scottish Index of Multiple Deprivation (SIMD) 2012 score is a non-linear numerical scale of material deprivation based on postcode of residency. The SIMD ranges from 0.94 to 89.89 with higher numbers representing residential areas with the most material deprivation and socioeconomic inequality. The score is generated by 38 indicators in 7 weighted domains including income, employment, health, education, crime, housing and access to services (Scottish Index of Multiple Deprivation (Public Health Scotland Data and Intelligence), 2012).

Z-score of birthweight was calculated in cells based on gestational age, sex and parity using LMS tables constructed from a reference population of all Scottish births from years 1998-2003 (Bonellie et al., 2008).

Large for gestational age (LGA) is defined as an infant born with birthweight above the 90th centile (adjusted for gestational age, sex and mother's parity) and is based on the same reference Scottish population from 1998-2003 (Bonellie et al., 2008).

Small for gestational age (SGA) is an infant born with an adjusted birthweight below the 10^{th} centile.

Stillbirth is legally defined in the 1992 amendment of the Births and Deaths Registration (Scotland) Act 1965 as a child born after 24 weeks gestation which did not breathe or show signs of life at delivery (Registration of Births, Deaths and Marriages (Scotland) Act. Sect. 56, 1965). Prior to 1992, stillbirth was defined as 28 weeks. The crude stillbirth rate is used in this study and is presented as a rate per 1,000 total births.

Perinatal mortality is the combination of stillbirths and infant deaths which occurred in the first seven days of life. This is also presented as a rate per 1,000 total births.

Hypertensive disorders diagnosed in pregnancy are of interest in this study, but were not included in full analysis in these studies due to limited reliability of data. Audits have shown that these disorders are underreported by up to 40% in SMR02, and any coding that did exist would be difficult to establish the diagnostic criteria used (Intelligence).

2.1.4.4 Statistical analysis of pregnancy outcomes according to diabetes diagnosis

Analysis was performed using SPSS version 22.0 (IBM Corp., USA).

When analysing mode of delivery, gestational age at delivery and birthweight variables, only singleton deliveries are included since multiple births could have significant influence on these parameters unrelated to diabetes status. The same influence can be seen with higher risk of intrauterine death in multiple births, however, stillbirth and perinatal mortality are uncommon and serious outcomes, and so all births are included in this analysis.

Data are expressed as mean+SD, or percentages as appropriate.

To compare demographics or pregnancy outcomes in mothers with T1DM, T2DM or without diabetes, ANOVA with post-hoc testing for between group differences is used for continuous parameters, and χ^2 used for categorical variables.

When analysing time trends, logistic regression models are used that include terms for diabetes diagnosis (T1DM, T2DM or no diabetes), time (defined by the year of interest) and any interaction between the two. Binomial distribution is used to calculate 95% confidence intervals.

2.1.5 Diabetes and stillbirth risk factors methodology

The same linked databases, SMR-02 and SCI-Diabetes are used for this study. At the time of beginning this study, the latest excerpt included linked data to 30th June 2016. Data over a longer period from 1st April 1998 to 30th June 2016 is therefore analysed.

The main aim of is to define the risk factors associated with a stillbirth outcome in pregnancy complicated by diabetes. The effect of maternal and fetal characteristics, and regional variation in factors linked to stillbirth are explored.

2.1.5.1 Study population

In this study, only singleton deliveries to mothers with pregestational T1DM or T2DM are included. As in the first study, inpatient episodes that resulted in delivery of an infant at or beyond 24 weeks of gestation are included.

Information relating to maternal demographics, infant characteristics at delivery and data relating to glycaemic parameters measured during pregnancy are included. Comparisons of these factors between pregnancies that ended in stillbirth and those which resulted in a livebirth are made to better understand the demographics of diabetic pregnancies at risk of severe adverse outcome.

Analysis according to unit where pregnancy was delivered is also performed to assess for any differences in regional outcomes suggestive of inter-regional variability in population or obstetric practice. Regional analysis is performed in two ways. Firstly, exploring differences according to health board area in which delivery occurred, and secondly exploring the size of delivering unit defined

according to the average number of deliveries per annum of women with diabetes.

2.1.5.2 Health board regions in Scotland

Scotland is divided into 14 geographical areas whose healthcare policy and spending is overseen by a regional health board. Central government funding is provided to these health boards who then have the responsibility of allocating resource and financing regional healthcare services for their population. Figure 2.2 demonstrates the 14 health board regions. They vary significantly both in terms of population size and demographic. For example, NHS Orkney is the smallest health board with a population of just over 20,000 people and covers a rural, off-shore island whilst NHS Greater Glasgow and Clyde is the largest health board serving a population of 1.2 million in a smaller, densely populated region in West Central Scotland (Scotland's health on the web, NHS Scotland). NHS Highland covers a much vaster area at almost 40% of Scottish land mass, but serves a population approximately four times smaller than that of NHS Greater Glasgow and Clyde.

Regional health care setups may differ in terms of access to specialist centres and experience of these centres in dealing with higher risk pregnancies. Whilst all have diabetes and pregnancy clinic setups, some of the more peripheral regions may decide to send particularly high-risk women to more central tertiary centres in the large Scottish cities. This is one of the reasons that migration of patients across health board regions may occur. Patients may also migrate due to patient choice of delivery centre or because resident close to boundary of two regions.

Taking into consideration potential differences in access and experience of specialist diabetes and pregnancy services across Scotland, and the potential for patient migration between across health board boundaries to receive obstetric care, I analysed the effects of health board area of delivery on outcome. The outcomes of interest were specifically stillbirth rates, birthweight Z-score and gestational age at delivery. Three of the smallest health boards (Western Isles,

Orkney and Shetland) are excluded due to low numbers of deliveries to women with diabetes determined either by <5 deliveries to women with diabetes per annum pre-analysis (1 region), or exclusion post-analysis (2 regions) when visualised mean outcome and very large confidence intervals. Health boards are represented as anonymised regions A-K.

2.1.5.2.1 Outcomes by size of unit of delivery

Obstetric units across Scotland will have varying degrees of experience in delivering women with pregnancy complicated by diabetes. Some of the larger city hospitals can expect to deliver up to 50 infants to mothers with diabetes per annum, whilst some of the smaller units will deliver less than 5 per annum. Size of delivering unit is therefore categorised according to number of women with diabetes delivered there per annum across the study period. They are defined as <5, 5-9, 10-19, 20-29 and 30-50 deliveries/annum. Those units in which number of deliveries are so small (<5 deliveries across the entire study period) are excluded. These centres contributed <1.8% of all deliveries (126 deliveries in total).

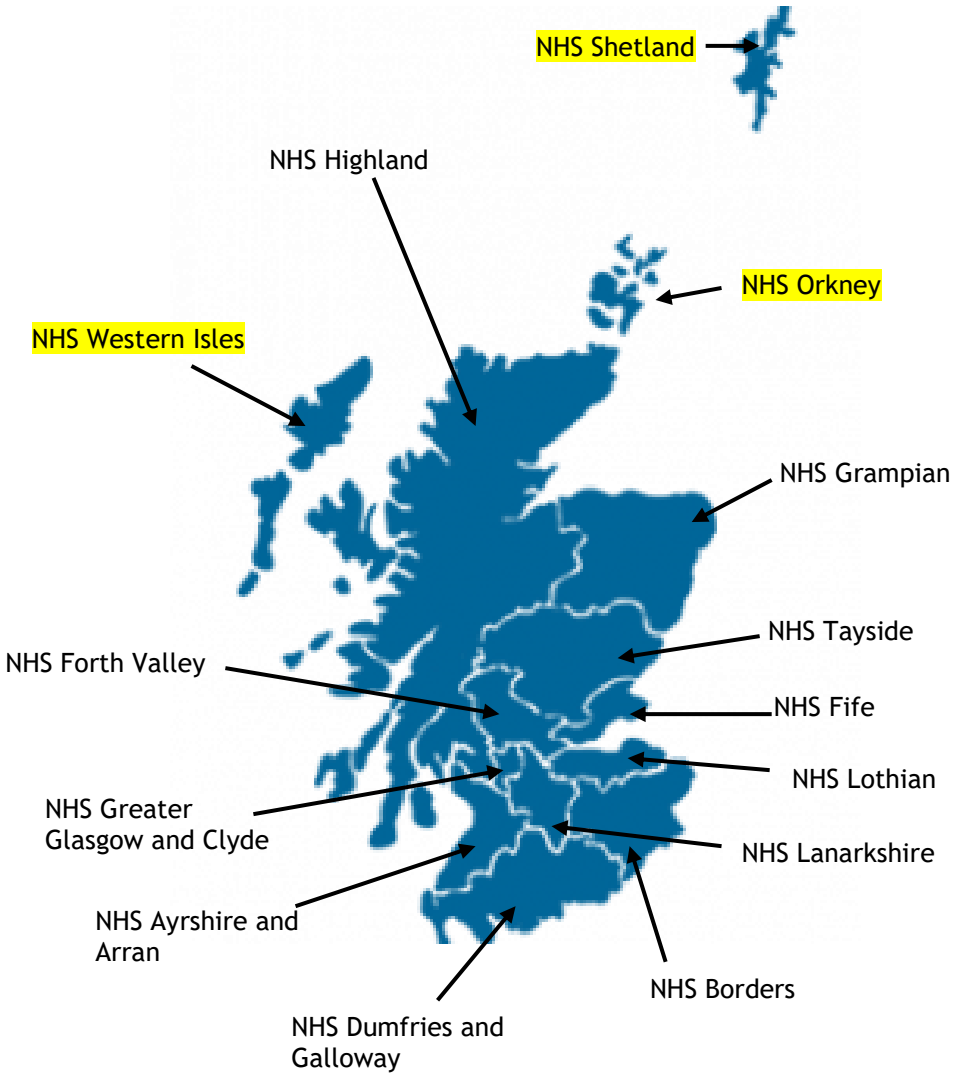


Figure 2.2: Health board geographical regions of Scotland

This map of Scotland outlines the geographical areas covered by the 14 regional health boards. Each health board boundary is depicted by white boundary lines and is labelled according to the responsible health board. Highlighted areas depict those health-boards excluded from regional analysis.

2.1.5.3 Definitions

The definitions for stillbirth, birthweight Z-score, LGA and SGA are the same as those described in section 2.1.4. SIMD2012 score is used again to estimate material deprivation.

Maternal BMI is defined as the last recorded BMI in SCI-diabetes within the six-month period preceding conception. Conception data are calculated using the date of delivery minus the gestational age at delivery.

Maternal HbA1c data available from each trimester are analysed. The last available HbA1c within each time frame is used. Pre-2006, labs were not routinely DCCT-aligned and so some regional variation in assays may be expected prior to that time period.

Pre-pregnancy is defined as up to six months preceding estimated conception.

First trimester is defined as day 1-90 (up to 12+6 weeks) of pregnancy inclusive.

Second trimester is defined as day 91-188 (13+0 to 26+6 weeks) of pregnancy inclusive.

Third trimester is defined as day 189 (27+0 weeks) to delivery

2.1.5.4 Statistical analysis of risk factor differences between stillbirth and livebirth pregnancies

Data were analysed using Statistical Analysis Software (SAS) v 9.4 (Cary, NC, USA). Risk factors and obstetric outcomes are presented separately for diabetes diagnosis (T1DM or T2DM).

Outcomes of interest in stillbirth and livebirth pregnancies within each of these groups are presented as a mean $\pm$ SD or frequency as appropriate. Crude stillbirth rates (per 1,000 deliveries) are presented. Gestational age of stillbirth

is defined as the gestational age at delivery of the infant, but death may have preceded this time.

For comparison of maternal and infant characteristics between live and stillbirth groups, generalised mixed linear models are used, with a random effect incorporated for mother ID. This was recommended following peer-review of the data to account for any effect of mothers having more than one pregnancy in this time frame, and contributing intrinsic but unmeasured stillbirth risk from the mother. The majority of women had one or two pregnancies only in this time period (67% one pregnancy, 94% one or two pregnancies).

Analysis of regional variation in stillbirth rates combines data for both T1DM and T2DM pregnancies due to low absolute numbers. Analysis of regional and unit size variation of birthweight and gestational age at delivery outcomes are analysed separately for T1DM and T2DM. Analysis is performed using logistic regression analysis for categorical outcomes, and general linear model for continuous variables.

For data relating to timing of delivery and stillbirth risk at each gestational week, crude stillbirth rates with a denominator of number of ongoing pregnancies at that particular week of gestation are used.

For all analysis, statistical significance is assumed if $p<0.05$.

2.2 Maternal vascular function in gestational diabetes studies

2.2.1 Aims

As described in chapter 1, women with diabetes in pregnancy are significantly more likely to suffer conditions associated with vascular dysfunction, such as in preeclampsia. Glucose lowering in pregnancy reduces the risk of preeclampsia but the mechanism of this is not clear. I aim to explore whether GDM is associated with impaired maternal and placental vascular function, and whether treatment of blood glucose in later pregnancy alters vascular function. This chapter focuses on the maternal vascular function study methodology, although participants were recruited for wider participation in combined maternal and placental vascular studies (chapters 5 and 6).

The aims of the maternal vascular studies are to:

1. **Demonstrate if maternal endothelial function (as measured by FMD brachial artery) is impaired in pregnancy complicated by GDM compared with non-diabetic pregnancy**

2. **Demonstrate if maternal endothelial function (as measured by FMD brachial artery) changes with advancing gestation**

3. **Determine associated effects of glycaemia on maternal endothelial function (as measured by FMD brachial artery)**

4. **Determine differences in circulating maternal angiogenic biomarkers in women with GDM compared to non-GDM**

A prospective, observational cohort study was designed to assess these objectives.

2.2.2 Ethical approval

All work pertaining to the maternal and placental vascular studies in this thesis received ethical approval from Berkshire B Regional Ethics Committee, and local Research and Development approval from the sponsor NHS Greater Glasgow and Clyde.

2.2.3 Study Population

Women were recruited from the Princess Royal Maternity Hospital in Glasgow, and the Queen Elizabeth University Hospital Maternity outpatient departments in Glasgow.

The study was designed to include three groups of women and were recruited according to:

1. 30 women with GDM diagnosed according to IADPSG and national SIGN guidelines (fasting plasma glucose >= 5.1 mmol/L and 2-hour post 75g glucose load level of >= 8.5 mmol/L).

2. 15 women who had been referred for OGTT with risk factors for GDM but who had a normal OGTT result by IADSPG criteria (CONTROL 1)

3. 15 women with no risk factors for gestational diabetes (CONTROL 2)

Since risk factors for GDM such as obesity may be seen to influence vascular function independent of diabetes, two control groups (risk factor and healthy controls) are included as comparators for the GDM group.

Women in Scotland are referred to the maternity hospital outpatient department for OGTT between 24-28 weeks if they have risk factors for GDM, defined in table 2.3 (Management of Diabetes (Scottish Intercollegiate Guidelines Network), 2017). Women who were referred for routine GDM screening were considered for this study. For the healthy control group, these women would

not routinely be offered OGTT screening as part of routine clinical care. These women were instead recruited at their routine 20-week anomaly ultrasound scan, following a normal ultrasound report. Markers of glycaemia (fasting plasma glucose, HbA1c and fructosamine) were measured in all women at study visit 1 to account for lack of OGTT screening in this group.

Table 2.3: Risk factors that should prompt referral for GDM screening in Scotland

BMI>30 kg/m^2
Previous GDM
First degree relative with diabetes
Previous macrosomic baby
High risk ethnicity (South Asian, Middle Eastern, Black Caribbean).

At initial study setup, it was intended those women with GDM and risk-factors for GDM would be recruited from between 24-28 weeks when GDM screening should take place, with the aim that the first visit should be completed between 24-28 weeks. However, after the study had commenced it became apparent that due to variations in timing of OGTT and obstetric clinic schedules, women diagnosed prior to this time, and slightly later were being missed. We amended the protocol during the study to allow entry of women up to 32 weeks and 6 days provided that the first study visit could be completed in this time.

Obstetric ultrasound and OGTT clinic lists were screened by the research midwifery team and eligible women approached in the outpatient department and provided a patient information leaflet. If women were open to consideration of the study, they were asked to read the patient information leaflet at home and leave contact details. Women who were agreeable were given at least 24 hours to consider the study before telephone contact to confirm if they wished to participate.

2.2.3.1 Inclusion and exclusion criteria

Women were deemed eligible for this study if they were:

- Aged 18+ years with singleton pregnancy
- Normal 20-week anomaly scan
- Able to provide written, informed consent

They were excluded from participation if met any of the following:

- Aged <18 years of age
- Multiple pregnancy
- Pre-existing diabetes (not including previous gestational diabetes)
- Pre-existing hypertension diagnosed prior to study entry
- Women on potentially vasoactive medications, or other medications that may be deemed by the investigator to potentially interfere with study procedures or results (excluding Aspirin, levothyroxine, folic acid, vitamins)
- Significant pre-existing illness, particularly if known vascular effects
- Congenital anomaly
- Identified placental pathology
- Unable to give informed, written consent
- Unable to understand written English language

2.2.4 Study visits

2.2.4.1 Study visit schedule

Four antenatal study visits were scheduled to coincide with routine antenatal clinical appointments to maximise participant retention. The initial study protocol was designed such that the baseline visit would occur shortly after OGTT performed as part of routine clinical care between 24-28 weeks, and immediately prior to their routine obstetric clinics. Due to local variation in timing of OGTT and obstetric clinic schedules, the protocol was later extended to allow women to enter the study up to the 32 weeks and 6 days gestation.

Visit 1 largely occurred between 24-30 weeks gestation with one participant outlying with entry to study at 220 days gestation. Visit 2 took place between 32 and 33 weeks of pregnancy. Visit 3 two weeks later between 34 and 35 weeks of pregnancy and visit 4 a further two weeks later between 36 and 37 weeks of pregnancy.

All study visits and study procedures were conducted independently by principal investigator (Sharon Mackin) in a temperature-controlled room in the Queen Elizabeth University Hospital Clinical Research Facility, Glasgow.

2.2.4.2 Study visit procedures outline

Figure 2.3 outlines study procedures according to visit number.

Visit 1: Participants were asked to attend after an overnight (minimum 8 hour) fast and to have refrained from caffeine and nicotine containing products. Clinical information and maternal demographics were collected using a combination of patient interview and routine electronic health records (Clinical Portal™ and BadgerNet™). Data were collected on current gestation, OGTT results in GDM and risk factor groups, previous and current pregnancy details (including complications), past medical history, current medications and smoking status. Data were recorded on the case report form which is included in Appendix 1.

Women were weighed on calibrated clinical-grade scales (Seca™ Germany), and details of their early pregnancy (obstetric booking appointment) weight was recorded from BadgerNet clinical database. Participants underwent BP measurement and FMD brachial artery (right arm) analysis as detailed in section 2.2.5.

Venepuncture was then performed with samples taken in EDTA, serum and sodium fluoride vacutainer tubes (see section 2.2.6 for details of sample processing and storage). These samples were later analysed for measures of glycaemic control (HbA1c and fructosamine), insulin resistance (HOMA-IR), lipid profiles, inflammatory cytokines IL-6 and IL-1ß and circulating angiogenic factors sFlt-1, PlGF and TNF-α.

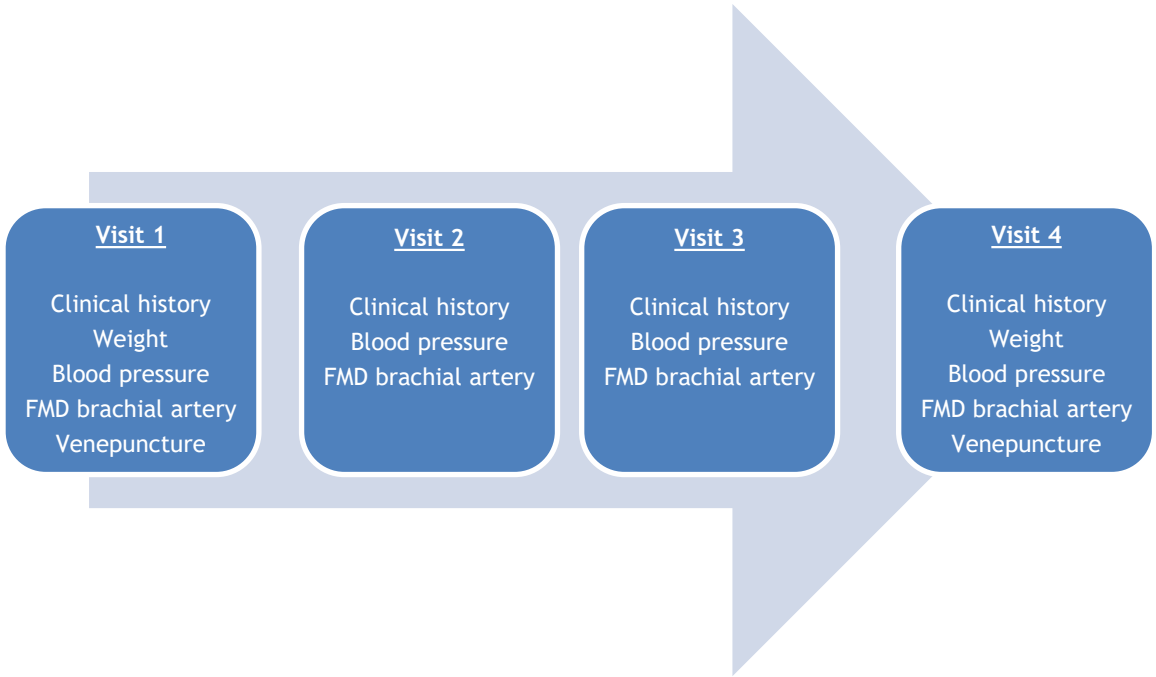


Figure 2.3. Study visit procedures according to study visit number

Study visit procedures listed according to study visit number. Visit 1 occurred at 24-30 weeks gestation and included clinical history, weight and BP measurement from participants, FMD of brachial artery and venepuncture performed for maternal markers of glycemia, insulin resistance, lipids and circulating angiogenic factors. At visit 2 (32-33 completed weeks gestation) and Visit 3 (34-35 completed weeks gestation), participants updated on changes to their clinical history and medications since the previous visit, and underwent BP and FMD brachial artery measurement. At visit 4, participants updated on changes to their clinical history and medication since their last visit, had their weight, BP and FMD brachial artery measurements performed, and underwent venepuncture. Bloods taken were again processed and stored for later analysis of maternal glycemia, insulin resistance, lipids and circulating angiogenic factors.

Visit 2 and 3: Participants attended fasted (minimum of 4 hours as no fasting bloods required) but must have avoided caffeine and nicotine that day. Clinical information was collected on current gestational age, presence of new medical/obstetric diagnoses and current medications. Participants with GDM were recording home capillary blood glucose readings as part of routine clinical care and this information was collected. BP and FMD of brachial artery were again measured.

Visit 4: Participants attended following an overnight fast (minimum 8 hours) and asked to avoid nicotine and caffeine products prior to their visit. Clinical information relating to new obstetric and medical problems, and medication use were updated. Women were again weighed on calibrated scales (Seca™, Germany). BP and FMD of brachial artery were measured as before. Venepuncture was again performed with samples taken in EDTA, serum, and sodium fluoride vacutainer tubes. These were processed and stored and later analysed for the same biomarkers as visit 1 samples.

2.2.5 Flow mediated dilatation of brachial artery protocol

2.2.5.1 FMD brachial artery procedure

FMD of brachial artery was chosen as a non-invasive, widely accepted and validated measure of assessing endothelial function in pregnant and non-pregnant subjects (Foo et al., 2017). The procedure involves measuring shear stress-induced vasodilation (as a % from baseline) induced by laminar blood flow post-arterial occlusion. Shear stress induces NO release from endothelial cells which should lead to vasodilation. Lower vasodilating capacity is associated with endothelial dysfunction and cardiovascular risk (Celermajer, 1998).

The procedure was performed in a temperature-controlled room (23-26^{0}C) using the semi-automated FMD device UNEX EF 38G by a single researcher (Sharon Mackin). Figure 2.4 shows the UNEX EF device. FMD procedures were conducted according to Task Force guidelines for the ultrasound assessment of vasodilation of the brachial artery (Corretti et al., 2002).

FMD measurement is a highly specialised skill that can be prone to significant inter-user variability, particularly in cases where training is inadequate, the user is inexperienced or stringent study procedures are not followed to minimise vasoreactivity. The UNEX EF™ device is a novel piece of equipment that uses a stereotactic B mode ultrasound probe and in-built wall detection software to calculate semi-automated FMD measurement. The semi-automated equipment was designed to minimise user error and variability in image interpretation seen in manual FMD measurement (Corretti et al., 2002). I was trained and appraised locally by more experienced users of FMD, and practiced on healthy volunteer colleagues for a three-month period prior to the first study visit.

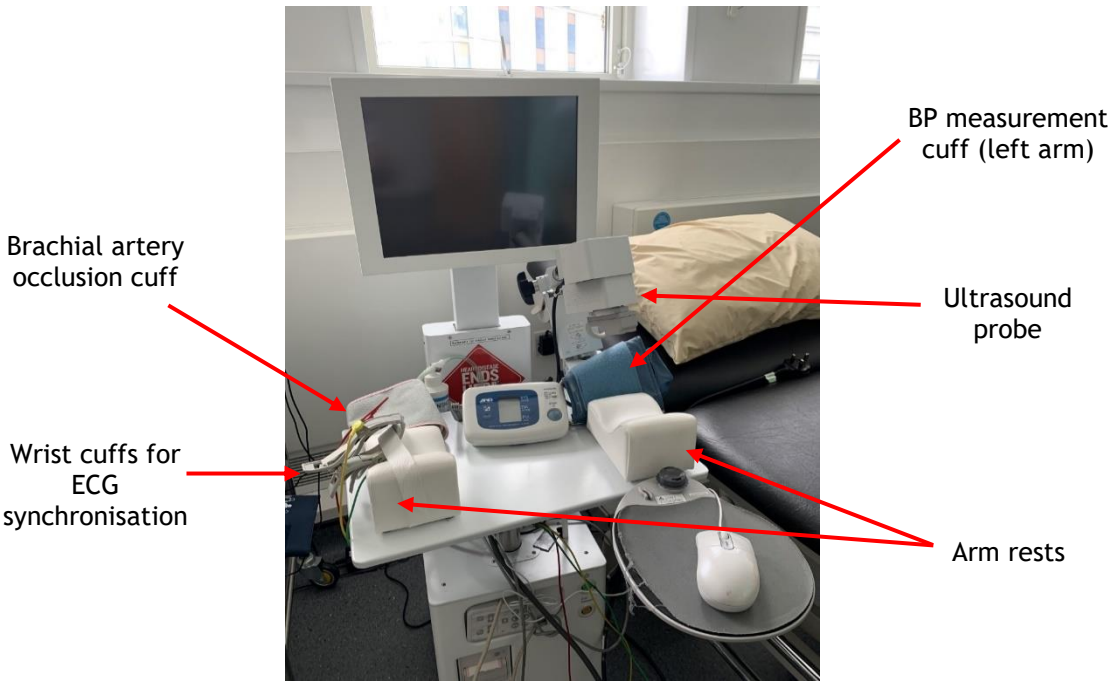


Figure 2.4: UNEX EF flow mediated dilatation technology

Participants lay with their arm placed in a relaxed position across both arm rests. Arm rests are attached to the stand using magnets to minimise movement. Two cuffed metal electrode bracelets are attached to each wrist to allow detection and synchronisation of the device with the cardiac cycle. There is automated BP cuff which should be placed on the upper arm on the opposite side to the brachial artery being imaged. There is a second inflatable cuff that is placed on the forearm on the same side that FMD measurement is being measured, and will be inflated to above systolic pressure to cause arterial occlusion. There is a stereotactic linear ultrasound probe that can be locked in position after satisfactory images of the brachial artery and imaging are obtained, but prior to beginning FMD measurement.

Participants attended the clinical research facility fasted (minimum of four hours) and having avoided all caffeine and nicotine-containing products that day. They were allowed to relax in a seated position for fifteen minutes whilst asked questions relating to their medical, obstetric and medication history.

Participants then lay on a bed with the head raised to an approximate $15\text{-}30^0$ angle (to minimise vena cava obstruction from a gravid uterus), and their right arm rested perpendicular to the body on the UNEX EF arm rests.

An automated BP cuff (connected to the UNEX EF device) was placed on the upper LEFT arm, and two cuff electrodes placed on both wrists to allow synchronisation of the FMD device to the cardiac cycle. A second inflatable cuff was applied to the extended RIGHT forearm.

The automated BP cuff attached to the LEFT arm was inflated and the BP result obtained was automatically relayed to the UNEX EF machine.

Using the ultrasound probe of the UNEX EF, the brachial artery was imaged 5-15cm above the antecubital fossa. The ultrasound probe was manually manoeuvred to provide a clear longitudinal image that allowed identification of the near and far intraluminal artery walls (intima), and two short axis images (proximal and distal) (see Figure 2.5). The ultrasound probe was then locked in position and the participant asked to remain as still as possible for the remainder of the procedure. Electronic markers were placed in the centre of the vessel on short axis views, and the UNEX EF device then used automated wall tracking technology to further improve probe position and optimise intraluminal vessel views. A resting intraluminal brachial artery diameter was then recorded (from intima to intima) by UNEX EF over 10 consecutive heart beats, and an average of these provided as the “rest diameter”.

The inflatable cuff on the RIGHT forearm was then inflated to 50mmHg above the participants measured systolic BP (SBP) for a total of five minutes, with the intention of occluding flow in the brachial artery for this duration. Participants

were encouraged to remain still throughout. After four minutes occlusion time, the UNEX EF device, began automatic retracking of the intraluminal vessel diameter in preparation for cuff deflation and subsequent measurements. The intraluminal diameter in the 60 seconds pre-cuff deflation was recorded by UNEX EF as "base diameter." Following cuff deflation, the UNEX EF device tracked beat to beat intraluminal diameter for two minutes. The time and diameter reached at maximum vasodilatation post-cuff occlusion was recorded.

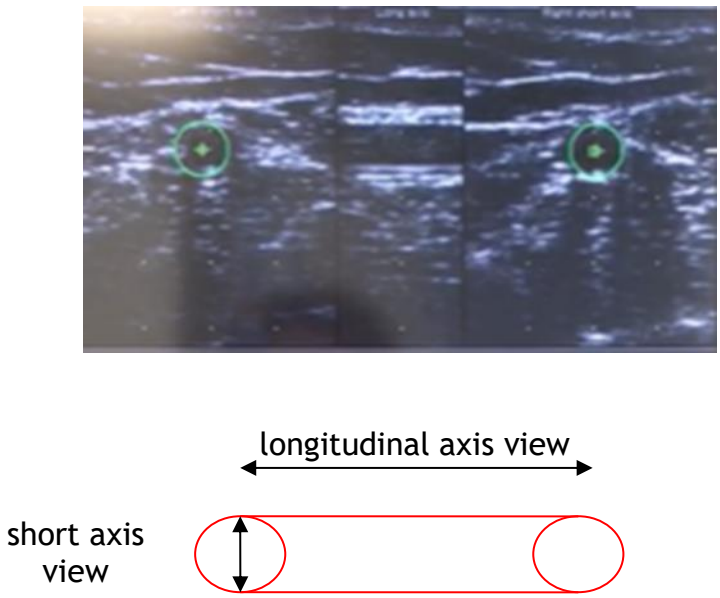


Figure 2.5: Example of typical longitudinal and short axes images on UNEX EF 38G.

The longitudinal and short axes are shown simultaneously on the 15" UNEX EF 38G screen. The longitudinal image is placed centrally and should show clearly lineated near and far internal vessel walls. Left and right short axis are shown accordingly on each side of the longitudinal image. The green circles on short axis views highlight the markers placed centrally by the user over the vessel. The UNEX EF 38G uses these markers to assist location of the vessel under the probe and optimise vessel images using automated tracking.

This image was taken by principal investigator Sharon Mackin.

2.2.5.2 FMD brachial artery automated analysis

The UNEX EF provides an output with automatically calculated FMD. However, the UNEX EF reports variable FMD outputs dependent on the automated interpretation of images. Scrutiny of automated results revealed that UNEX EF would also automatically calculate FMD despite some very poor quality images.

UNEX EF offers a series of FMD outputs termed:

- **C-FMD**: A measurement comparing pre-cuff deflation "base diameter" with maximum diameter based on longitudinal image measurements. Calculated as

$$\frac{maximum\ diameter - base\ diameter}{base\ diameter} \times 100\%$$

- **FMD:** A measurement comparing pre-cuff inflation "rest diameter" with maximum diameter based on longitudinal image measurements. This measurement provided alongside "C-FMD". Calculated as:

$$\frac{maximum\ diameter - rest\ diameter}{rest\ diameter} \times 100\%$$

- **FMDs:** A measurement comparing pre-cuff inflation "rest diameter" with maximum diameter based on short axis images. Often used by the UNEX EF device if longitudinal images were felt be of inadequate quality for auto-interpretation of FMD. Calculated as:

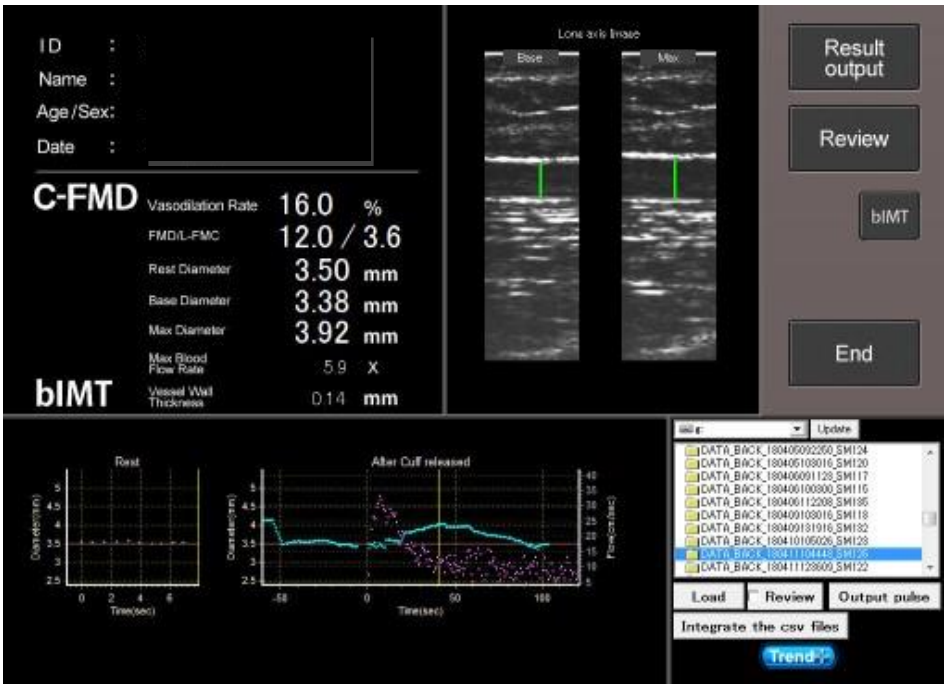
$$\frac{maximum\ diameter - rest\ diameter}{rest\ diameter} \times 100\%$$

Figure 2.6 A-D shows examples of the variable automatic outputs from UNEX EF.

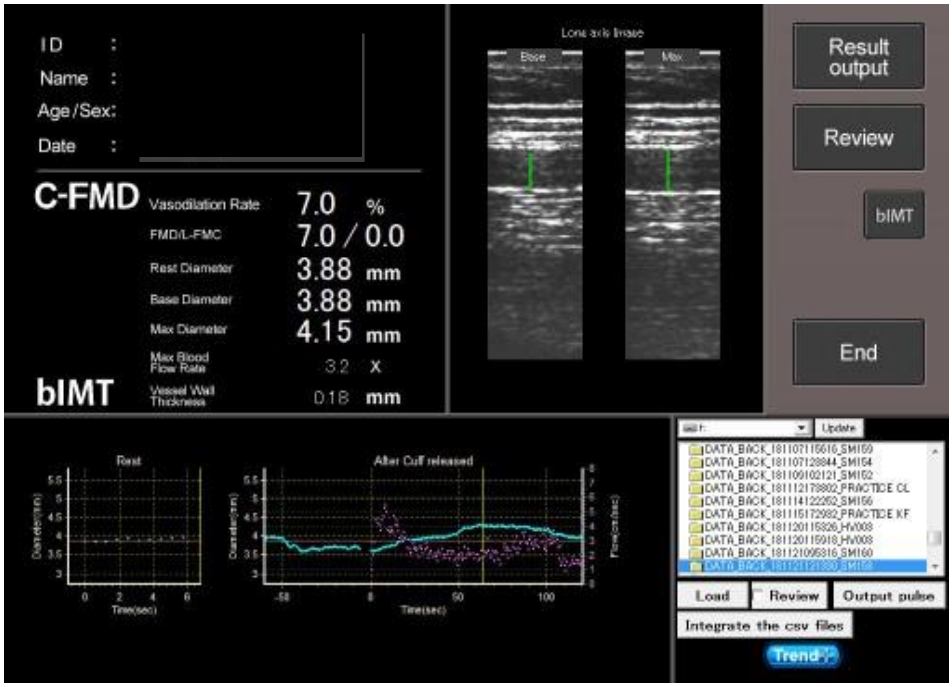
Due to variability in automated reporting methodologies used by UNEX EF, I identified a need to assess the reproducibility of UNEX EF reporting. I taught three students on the use of automated UNEX EF measurement and subsequent manual analysis, and supervised them in a study validating the semi-automated UNEX EF measurements in healthy volunteers. In this study, we compared reproducibility of two semi-automated FMD readings taken 20 minutes apart and subsequent reproducibility with manual reanalysis. This confirmed my hypothesis that manual measurements had good reproducibility (ICCC 0.679 with low CV 12%) compared to automated measurements (ICCC 0.103 with high CV

46%) (Dobbie et al., 2020). Furthermore, a quarter of scans were non-diagnostic in the hands of these inexperienced users supporting the need for a manual quality check on images prior to inclusion of UNEX EF data in any study. Given this superior reproducibility, I decided to perform manual quality assessment and measurement of FMD for all recordings in this study. I performed all manual analysis.

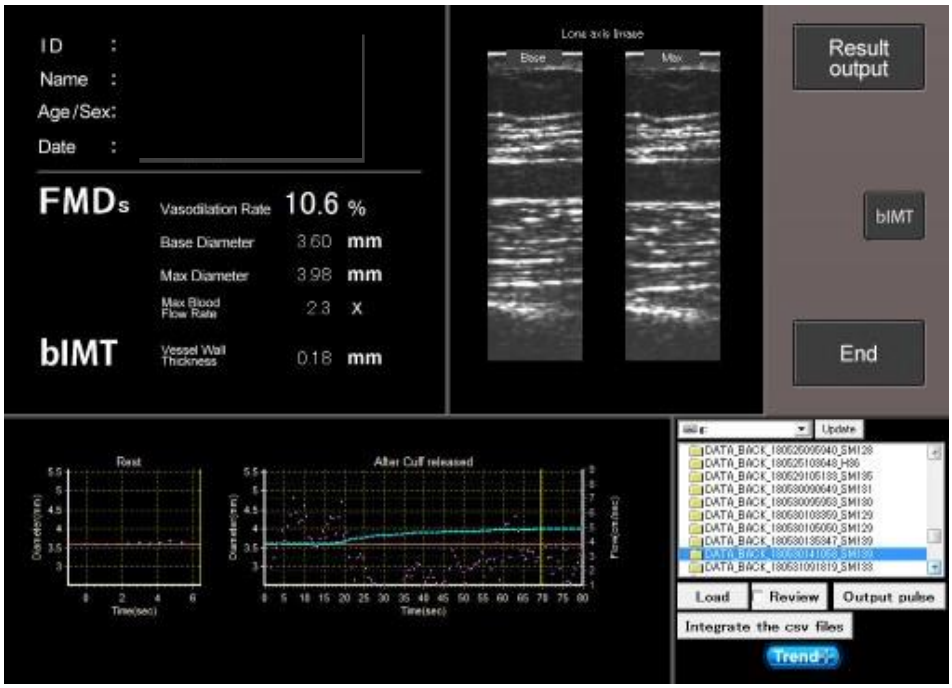
A



B



C



D

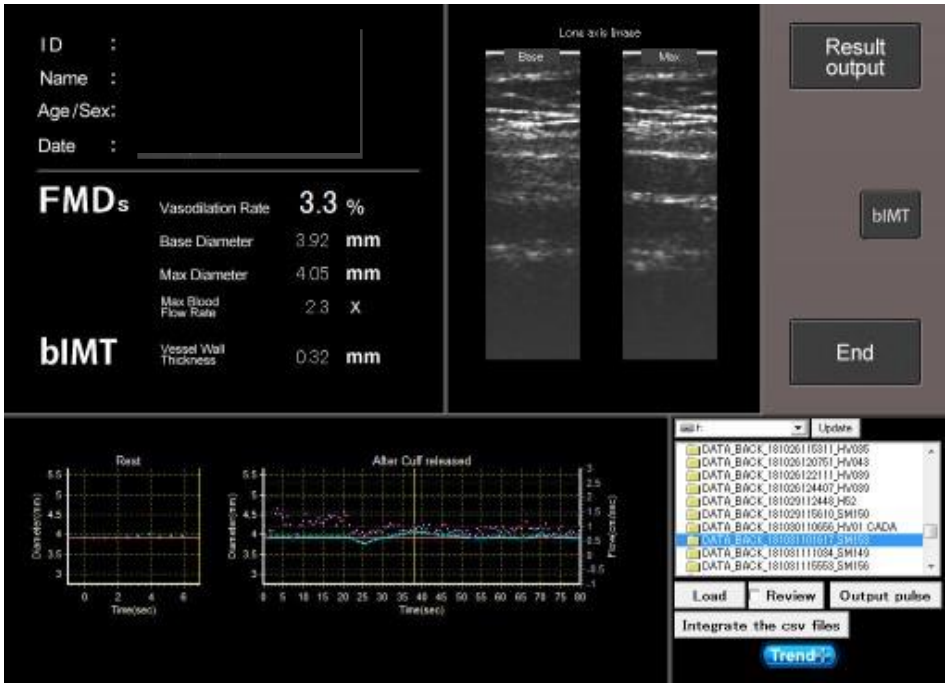


Figure 2.6: Examples of acceptable and inadequate quality FMD outputs from FMD UNEX EF 38G.

Each UNEX-EF output provides two still longitudinal images of what it has interpreted as base diameter and max diameter. The graphs on the bottom left of screen plot the intraluminal diameter (blue) in mm against time in seconds, whilst the detected flow rate through the vessel (cm/sec) is shown in the superimposed scatterplot (purple). Figure 2.6A shows a C-FMD output with clear near- and far internal vessel walls demonstrated, allowing measurement of FMD from measured parameters. Assuming that video playback had consistent images like this, I would consider it good quality to allow FMD measurement. Figure 2.6B shows a C-FMD output generated

by the semi-automated UNEX-EF device, but on manual inspection it is clear that the device is picking up flow interference mid-lumen and interpreting this as vessel intima. The UNEX-EF generated FMD result is therefore likely inaccurate in this case. Figure 2.6C shows images from a recording that the UNEX-EF device felt had too poor quality to generate an FMD result from longitudinal images, and therefore reverted to calculating FMDs using short axis images. On manual inspection of the still images and playback of video, I felt that the intraluminal diameter was consistently visible and after manual reanalysis was happy to include this in the study. Figure 2.6D on the other hand shows very poor quality images where the UNEX EF has generated an FMD value based on short axis images. This image was excluded from any analysis.

2.2.5.3 FMD brachial artery manual analysis

I performed a manual quality check of each UNEX EF generated FMD measurement using UNEX EF PC analysis software (UNEX Corporation, Nagoya, Japan). This involved reviewing playback of all UNEX EF generated recordings and assessing quality of ultrasound images throughout the duration of experiment. FMD results were deemed of inadequate quality if the intima-to-intima interface was not readily identifiable in the pre-cuff occlusion rest period, or consistently throughout the two minute post-cuff deflation period. Those considered of poor quality were excluded from further analysis.

To achieve consistency in methodology, all remaining images were manually analysed. The rest diameter was manually measured from images obtained pre-cuff inflation. To measure the maximum diameter, the near and far vessel walls were identified on immediate post-cuff deflation images. Once marked on the UNEX EF images, the machine was instructed to “overwrite” and the images played forward. The software used these pre-identified vessel wall markers in combination with wall tracking technology to overwrite the intraluminal diameter. Images were then re-reviewed by me on playback to ensure that it was consistently measuring intraluminal diameter. If deemed acceptable, then the diameter and time at which maximum dilatation first occurred was noted. FMD was then calculated as the % difference between the rest and maximum diameter.

2.2.6 Biochemical measures

2.2.6.1 Sample preparation and storage

Blood samples were collected from patients on visit 1 and visit 4. Venepuncture was the last study procedure performed at these visits to minimise any effects on FMD measurement from stress or in the unlikely event of accidental arterial puncture. For the visits that required venepuncture, women attended following an overnight fast (minimum 8 hours). Approximately 20mL of blood was venesected into – 2x SST (serum), 2x EDTA and 1x Sodium Fluoride vacutainer tubes.

Samples were centrifuged within a couple of hours at a speed of 15000 rpm at 4^0C for 15 minutes. The supernatant was aspirated and aliquoted into 0.5mL volumes in cryotubes, clearly labelled as serum (SST), plasma (EDTA) or sodium fluoride. The buffy coat from the EDTA sample was aspirated and stored in a separate cryotube and the densest red blood cell portion aliquoted and labelled as red blood cells. Figure 2.7 shows the graded layers of the EDTA sample after centrifugation. All samples were stored in a -80^0C freezer until needed for assay. Thaw occurred immediately prior to any assay. A maximum of two thaws were allowed for any one aliquot.

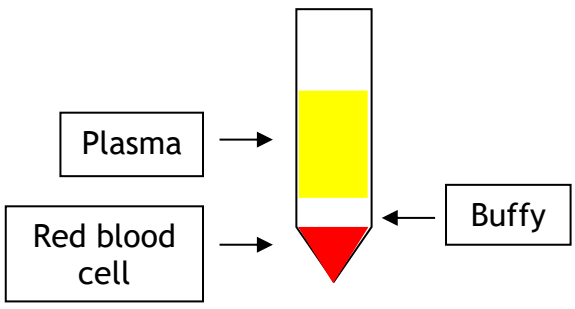


Figure 2.7: Sample layers after centrifugation of plasma samples

After centrifugation, plasma sample were aspirated into plasma, buffy coat and red blood cell samples before storage. This figure shows the typical layered sample after centrifugation. The yellow uppermost layer contains plasma fluid. The buffy coat contains white cells and other cellular debris. The red layer on the bottom contains red blood cells that were used for HbA1c analysis.

2.2.6.2 Assays – Glycaemic parameters and lipids

All glycaemic, lipid and albumin biomarkers were measured on the Roche Cobas C311 autoanalyser™. I assisted Elaine Butler (laboratory technician at the Institute of Cardiovascular and Medical Sciences, University of Glasgow) with these assays.

Plasma EDTA samples were used to measure fasting insulin levels (samples from patients on insulin were excluded from this) using a Roche Cobas™ electrochemiluminescence immunoassay.

HbA1c was measured on red blood cell samples using the Tina-quant™ HbA1c assay from Roche Cobas™.

Serum samples were used for lipid profiling (cholesterol, HDL and triglycerides), fructosamine using colorimetric assays from Roche Cobas™ (namely these were Cholesterol 2 gen, Triglycerides, HDL-Cholesterol 4 gen and Fructosamine kits) and an immunoturbidimetric assay for Albumin (AlbT2 kit from Cobas™).

Sodium fluoride samples were autoanalysed using UV absorption assay to measure glucose (Glucose HK 3 kit from Cobas™).

Further details on the properties of these assays can be found in appendix 2.

2.2.6.3 Assays – Angiogenic and inflammatory markers

sFlt1, PlGF and TNF-α concentrations in serum samples were using multiple analyte immunoassay magnetic bead technology (Milliplex™) from Merck™. The inflammatory cytokines IL-6 and IL-1ß were assayed using the same technology. Dr Lesley Graham (Institute of Cardiovascular and Medical Sciences, University of Glasgow) taught me how to do these assays, and I performed them independently thereafter.

The specific kits used were the Milliplex™ Human Angiogenesis Panel 2 (HANG2MAG-12k™) for sFlt1, Human Cardiovascular Disease Magnetic Bead Panel

1 (HCVD1MAG-67k™) for PlGF, and the Human Cytokine/Chemokine Magnetic Bead Panel (HCYTOMAG-60k™) for TNF-α, IL-6 and IL-1ß.

Samples were prepared and incubated with detection antibodies on a 96-well plate according to the manufacturer's instructions. A full protocol for each kit can be found in appendix 3. Each assay plate included a set of "standard" samples (provided in kit with known concentrations) to allow generation of a standard curve. Five-parameter logistic regression (5-PL) was used to generate the standard curve, or in some cases a 4-PL curve in the case of an ambiguous curve fit with 5-PL. Samples were analysed in duplicate on the MAGPIX xPONENT 4.2 (Luminex™) analysis system. For each sample well, a Median Fluorescence Intensity (MFI) was generated by the MAGPIX xPONENT system Median Fluorescence Intensity (MFI). Mean MFI for each duplicate sample was calculated and a corresponding analyte concentration obtained from the standard curve. The coefficient of variation of the MFI was calculated as a proxy assessment of test accuracy and reproducibility, with only those samples with <20% variation included in ongoing analysis.

2.2.7 Statistical analysis

The study was powered for the primary outcome of detecting a difference in FMD brachial artery between GDM and control groups. Based on previous cross-sectional work suggesting measures of FMD in GDM (9.2+6%) and pregnant controls (17.6+8%) (De Resende Guimarães et al., 2014), a power calculation for the primary outcome of difference in FMD% at baseline suggest that 16 women in GDM and control groups would have power (1-ß)0.8 to detect a difference of 8% with α=0.05. Statistical analysis was performed using IBM SPSS™ v 22.0 statistics package for the majority of analysis in maternal vascular studies. GraphPad Prism™ v 8.0 was used to generate standard curves using logistic regression (5-PL or 4-PL depending on best fit curve) and corresponding analyte concentrations for the Milliplex™ biochemical analysis.

Maternal demographics are compared between GDM, risk factor and control groups using one way ANOVA for continuous variables, and χ^2 for categorical variables. For continual variables, normality was assessed using Q-Q plots and Shapiro Wilk tests.

Time trend analysis of BP and FMD measurements are assessed using linear mixed models with terms for diabetes diagnosis and gestational age (in days) included as fixed effects, and participant ID as a random effect. This approach was chosen to account for intra-subject variability in measurements, and to allow interpretation in the presence of missing data. No specific pattern was identifiable in BP and FMD trends, making random computation of missing results difficult.

For biochemical analysis that was not normally distributed (assessed by Q-Q plots and Shapiro Wilk test), median values are presented and differences between groups assessed by non-parametric tests.

For all analysis, statistical significance is assumed if p-value of <0.05.

2.3 Placental vascular function in gestational diabetes studies

Placentas were collected prospectively from the same cohort described in chapter 2.2. Ethical approval for this study was submitted and approved under the same application as in chapter 2.2. Approval was from Berkshire B ethics committee and NHS Greater Glasgow and Clyde Research and Development committee.

2.3.1 Placental collection

At study entry and following prospective informed consent for placental collection at delivery, participants had a clinical alert entered on their electronic health record (BadgerNet™) asking the clinical team to contact the

principal investigator (Dr Sharon Mackin) by telephone, when the woman was admitted for delivery. All methods of delivery were included, and clinical teams were asked to contact the researcher regardless of time of day.

Once aware that a participant had been admitted in labour or for planned delivery, the clinical team and principal investigator agreed further telephone contact at the following times to arrange timely placental collection.

- Primiparous participants – when had reached 10cm cervical dilatation and just prior to commencing the second "pushing" stage of delivery, investigator should be contacted to attend hospital for impending delivery.

- Multiparous – when reached 8cm cervical dilatation or beyond, investigator should be recontacted to attend hospital for impending delivery.

- At any point where patient was being transferred for emergency caesarean section, the investigator was recontacted to attend labour suite.

- For elective caesareans, the investigator attended hospital when expected to be taken to theatre.

This allowed collection of the placenta and umbilical cord blood immediately post-delivery with minimal impact on the clinical team.

2.3.2 Cord blood sampling

Venous cord blood collection was consented for at entry of the study. This was agreed for storage for any future studies including DNA storage. No planned studies on cord blood formed the basis of the initial protocol and do not form part of this thesis. Cords were double clamped at delivery and using a sterile needle, 10mL maximum of blood was aspirated from the umbilical vein and

transferred into SST and EDTA samples. It was centrifuged and aliquoted as in section 2.2.6.1 and stored at -80^{0}C. Participants had the option to complete the study but decline cord blood sampling.

2.3.3 Placenta sampling technique

In the laboratory, placenta were gently stripped of the chorionic membranes and cord and weighed on calibrated scales. The chorionic plate surface was grossly visualised for an area that contained small chorionic plate vessels, and a full thickness sample was cut from this area and placed in ice cold physiological salt solution (PSS: 0.25 mmol NaCl, 1 mmol KCl, 50 mmol $NaHCO_3$, 2 mmol KH_2PO_4, 1 mmol Glucose, 2.5 mmol $CaCl_2$). Details of salt solutions used in these experiments are in Appendix 4.

Using a sheet of acetate with 2x2cm squares marked on it, biopsies were taken from each quadrant 2cm distal to cord insertion. These were flash frozen in liquid nitrogen and stored at -80^{0}C for any future experiments. These samples are currently being used for microRNA experiments conducted as part of my current lectureship, but not as part of this thesis.

2.3.4 Chorionic plate artery (CPA) myography

2.3.4.1 CPA preparation and myograph mounting

From the first placental sample selected due to macroscopic visibility of chorionic plate vessels, chorionic plate arteries were dissected. The biopsy and vessels were continuously bathed in petri dish containing cold PSS solution.

Small third branch chorionic plate arteries (CPAs) were identified under a microscope. Typically, arteries had slightly thicker walls than corresponding veins and tended to lie on top of the corresponding vein. Using specialist microvascular dissection tools, the artery was cut free from the chorionic surface and trimmed of visible connective tissue. Care was taken at all times to minimise contact with the vessel.

Following dissection of CPAs, they were cut into eight 2mm sections. These sections were mounted on two taut 40µm wires in separate Danish Myo Technology wire myograph baths (AD Instruments™, UK) containing 5mL of PSS. At resting position, wires were separated by approximately one wire width, enough to ensure no contact with the other wire but not applying stretch to the vessel.

The baths were heated to a temperature of 37^0C and bubbled with $5\%O_2/5\%CO_2/N_2$ to mimic the low partial pressure of oxygen found in the fetal circulation (Wareing 2006). CPAs were allowed to equilibrate in this relaxed state for 60 mins at these conditions before undergoing a normalisation procedure using the AD Instruments UK™ normalisation module. Figure 2.8 shows wire myography setup.

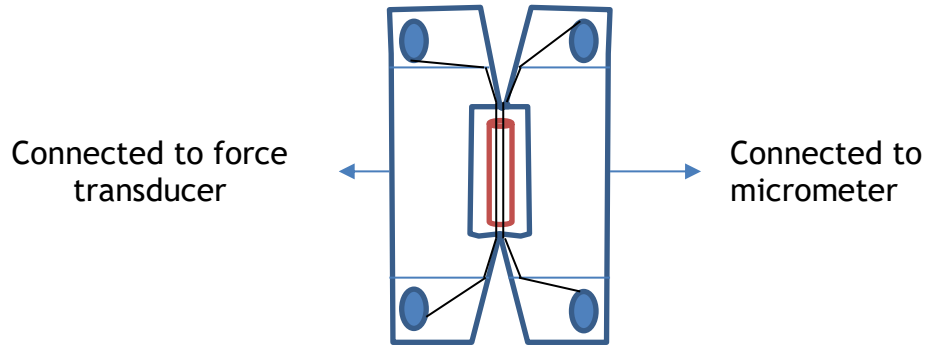


Figure 2.8: Wire myography setup

A dissected CPA of 2mm length is thread with two 40µm wires, which are pulled taut and screwed securely in place to the myograph. The resting position of the vessel, prior to normalisation, allows wires to lie as close to each other without touching. This is the resting state where no stretch is being applied to the vessel walls. There are two jaws of the myograph, one which is mobile and attached to a screw micrometer and the other which is fixed and connected to a force transducer. The screw micrometer is gradually adjusted to cause separation of the myograph jaws, and subsequent increasing stretch of the vessel. The other side is connected to a force transducer which measures the tension in the vessel wall. With each stretch of vessel, we can calculate the intraluminal diameter (2x40 µm plus the micrometer stretch from baseline). Intramural pressure can be calculated at each stretch using LaPlace's law (Pressure= Wall tension/(internal circumference/2π)).

2.3.4.2 Normalisation

Normalisation is the process of vessel stretching to an internal circumference that would achieve a baseline intramural pressure similar to those seen in vivo (Spiers and Padmanabhan, 2005). For systemic vessels, this would typically be 100 mmHg or 13.1 kPa. However, fetoplacental circulation is lower resistance than systemic vasculature (50 mmHg in the umbilical artery reducing to 30 mmHg as it transcends the chorionic plate and into the villus capillaries) (Wang and Zhao, 2010). Considering this, a target intramural pressure of 5.1kPa (approx. 40 mmHg) was chosen for these experiments, and is typical of methodology used in other placental myography studies. The internal luminal circumference of the vessel at this target pressure is called the IC_{100}. Once calculated, the vessels were relaxed to 0.9 of IC_{100} to optimise the interface between the actin and myosin filaments and contractile potential of vessels. $0.9L_{5.1kPa}$ is the term that will be used in the remainder of this thesis to describe these normalisation conditions.

2.3.4.3 Vessel viability

Following normalisation, CPAs were allowed to rest for a further 15 mins, before assessment of vessel viability by measuring contractile response to 5mL of 62.5 mmol potassium containing PSS (KPSS). CPAs which did not contract in response to KPSS, or who failed to achieve a vessel wall tension of 1 mN were considered non-viable, and not included in further analysis or experimentation.

For viable vessels, the contractile response to KPSS was allowed to plateau, before washout with 5mL PSS (x3) and settling back to their resting baseline. Contractility to 62.5 mmol KPSS was repeated three times with interspersed washouts. The maximum response of each CPA to 62.5 mmol KPSS contractions was recorded (mN).

2.3.4.4 Vasoconstriction - cumulative concentration response curve (CCRC) to U46619 (thromboxane mimetic)

Viable vessels again underwent washout (x3) and allowed to settle to baseline tension. Vessel contractility was then assessed in response to increasing concentrations of U46619 ((Enzo Life Sciences, UK) $1x10^{-10}$ M to $3x10^{-5}$ M). Cumulative concentration of U46619 in the myograph was increased in half log increments every 5 mins, or after maximal contraction to the preceding concentration had being achieved (whichever is longest). Maximal tension at each dose of U46619 was recorded and expressed as a percentage of the maximal response to KPSS. At the end of the U46619 CCRC, the vessels underwent a washout procedure (x3) and allowed to settle back to baseline resting tension.

2.3.4.5 Vascular vasodilatation – CCRC to bradykinin, sodium nitroprusside (SNP), calcitonin-gene related peptide (CGRP)

Following the above, CPAs were preconstricted with $3x10^{-7}$ M U46619 and maximal constriction recorded. Once maximal contraction had been reached, each vessel was exposed to cumulative increasing doses of either bradykinin, sodium nitroprusside (SNP) or calcitonin-gene related peptide (CGRP) as detailed below.

The initial protocol had been designed to investigate endothelial-dependent vasodilatation of CPAs to bradykinin ((Sigma-Aldrich, UK) $1x10^{-10}$ M – $3x10^{-5}$ M), and endothelial independent vasodilation to SNP ((Sigma Aldrich, UK) $1x10^{-10}$ M - $3x10^{-4}$ M) . Due to lack of endothelial dependent vasodilatory response from bradykinin in my initial samples, the protocol was amended to remove the bradykinin CCRC. This is a finding reported by other laboratories (Mccarthy et al., 1994, Dordea et al., 2013). A CCRC to human CGRP ((Tocris™, UK) $1x10^{-10}$ M -$3x10^{-4}$ M), a potent vasodilator with endothelial-dependent and independent mechanisms, was added instead.

To determine if CGRP effects were predominantly endothelial-dependent or independent in the CPAs, a separate CGRP curve was conducted following a 20-

minute incubation with the 100 μM of NOS inhibitor, N(ω)-nitro-L-arginine methyl ester (L- NAME) (Sigma Aldrich).

To prevent diminutive effects of repeated vasoconstriction and vasodilatation cycles and ongoing effects from previous agonist exposure, CPAs from each subject were split into groups so that each vessel was exposed to only one vasodilatory CCRC compound.

Doses for each CCRC were incremented in half-log concentrations at 5-minute intervals or whenever the maximal vasodilatory response had occurred to the preceding dose. Maximal response to each concentration was expressed as % of maximal constriction to $3x10^{-7}$ M U46619.

2.3.5 Statistical analysis

Differences between maternal and fetal demographics, and placental weight were compared using one way ANOVA or χ^2 as appropriate. IBM SPSS™ statistics v 22.0 was used for these analyses.

For the CCRCs, raw tension data for each vessel were analysed using LabChart Reader™ software v 8.1.13 (AD Instruments, UK). For each agonist and drug exposure, the mean tension was calculated for each subject. Mean agonist responses were then expressed as either maximal vasoconstrictive response relative to maximum KPSS response (%) or as maximal vasodilatory response relative to the maximum pre-constrictive response to $3x10^{-7}$ M U46619 (%).

A best fit cumulative concentration curve was then fitted on Graphpad Prism v 8.0 (GraphPad Software Inc™, USA) and comparison of curves between groups analysed using extra sum of squares F test. Maximal response to an agonist is expressed as Emax, whereas EC_{50} is the dose at which 50% of agonist response is achieved. Differences in Emax and EC_{50} between groups were assessed using one way ANOVA. Due to limitations in the numbers of control and risk factor placentas, analysis was conducted according to GDM and non-GDM groups. Statistical significance was assumed if $p<0.05$.

3 Chapter 3: Diabetes and pregnancy: maternal and neonatal outcomes

The work within this chapter has been published in Diabetologia, Jan 2018. 61(5):1081-1088 (Mackin et al., 2018).

3.1 Introduction

As discussed in chapter 1, diabetes confers significant additional risk in pregnancy. Rates of fetal overgrowth, operative and preterm delivery, and fetal and infant mortality are all increased compared to the population without diabetes. This is evident in a number of real-world population-level studies (Evers et al., 2004, Penney et al., 2003, Macintosh et al., 2006, CEMACH, 2005). The deleterious effects of hyperglycaemia on pregnancy are well documented in clinical trials (Metzger et al., 2008). Epidemiological data published since the 1990s have raised concerns that clinical teams are struggling to improve upon some adverse outcomes (Johnstone et al., 2006). This includes Scottish paper audits in 1998/99 and 2003/4 (Kernaghan et al., 2006, Penney et al., 2003) which show static infant mortality statistics. Despite marked improvement in perinatal mortality in previous decades, rates of stillbirth and perinatal mortality in pregnancy complicated by T1DM and T2DM remained stubbornly high at 3-5 times that of the background population (Evers et al., 2004, Fadl and Simmons, 2016, de Valk et al., 2006, Diabetes and Pregnancy Group France, 2003). Conversely, data from the England and Wales population suggest a marked reduction in stillbirth rates for women with diabetes over the last two decades. In England and Wales, stillbirth rates in T1DM have dropped from 25.8 (18.3 to 33.3) per 1000 live births in 2002/3 to 10.7 in 2013 , and from 29.2 (16.3 to 42.2) to 10.7 for women with T2DM (CEMACH, 2005, National Diabetes In Pregnancy Audit Report 2013, 2014). Importantly, this decrease has been sustained in their most recent 2018 audit (National Diabetes in Pregnancy Audit Report 2018, 2019). We lack more recent data to determine whether mortality outcomes remain high in mothers with diabetes in Scotland. Other outcomes are of interest also. A large single centre study in Scotland showed stable but high

mean birth weights in T1DM between 1960s and 1990s (mean birthweight Z-score 1.4) (Johnstone et al., 2006).

The focus of this study was therefore to analyse nationally collected data from Scotland to examine whether obstetric outcomes for mothers with T1DM or T2DM were improving in recent years, and to gain insight into the extent of ongoing challenges facing mothers with pregnancy and diabetes.

3.2 Materials and methods

3.2.1 Study population and clinical databases

The national inpatient maternity database, SMR02 was linked to the national diabetes database SCI-diabetes, and accessed as described in chapter 2.1.

From the SMR02 records, information on maternal and infant demographics, obstetric management and complications was collected for all females (>10 years of age) who delivered an infant at or beyond 24 weeks gestation in a Scottish hospital between 1st April 1998 to 31st March 2013 inclusive. Cross-linkage of this database to the national SCI-diabetes database allowed clinical data to be collected on diabetes diagnosis and clinical management for the same population. Gestational diabetes is not considered in this study due to database limitations.

3.2.2 Statistical analyses

Pregnancy outcomes in women by diabetes status (T1DM, T2DM or no diabetes) were compared using ANOVA or logistic regression with post hoc testing between groups (ANOVA) or by χ^2 test as appropriate. Trends for changes in outcome over time were assessed by ANOVA with terms for type of diabetes, time and interaction between these tested. Annual time periods were selected, except in the case of stillbirth and perinatal mortality rates where absolute number of cases were small with significant variability between years. Three-year rolling averages were used for assessing these time trends.

All analyses were performed using SPSS Version 22.0 (Armonk, NY: IBM Corp)

Data are presented for all deliveries for maternal variables, stillbirth and perinatal mortality (n=813,490) but confined to singletons for mode of delivery, gestational age at delivery including preterm birth and birthweight variables (n=801,263).

3.3 Results

3.3.1 Maternal population characteristics

813,921 deliveries were recorded in Scotland across the audit period, of which 38 were excluded due to unknown vital status of infant. Among these, 4683 (0.6%) were to mothers with pre-gestational diabetes, of which 3229 (69%) had T1DM for an average 13.3 years and 1454 (31%) T2DM for 3.3 years. Maternal and infant demographics are shown in table 3.1. A further 249 mothers were linked to SCI-diabetes with another diagnosis (including gestational diabetes, impaired glucose tolerance, maturity onset diabetes of the young) and were not considered further.

Mothers with T1DM were on average 0.4 years older and more likely to be nulliparous than the obstetric population without diabetes. By contrast, mothers with T2DM were 4 years older than the general obstetric population, had higher deprivation scores and were more likely to have had a previous pregnancy (table 3.1)

The absolute number of births per annum to mothers with T1DM increased from 1998/1999 to 2012/2013 from 205 to 264 deliveries per year, with a larger increase in T2DM from 59 to 110 deliveries per year. Both increases were statistically significant ($p < 0.001$) and suggested a 44% increase in deliveries to mothers with T1DM and a 90% increase in T2DM in fitted linear models over time. When represented proportionally to the number of total births in Scotland, T1DM and T2DM were both more prevalent in 2012/13 compared with 1998/99 (Figure 3.1).

Table 3.1 Maternal demographics according to pre-pregnancy diabetes status

	Type 1 diabetes	Type 2 diabetes	No pre-gestational diabetes[a]
Number of ongoing pregnancies after 24 weeks	3229	1452	808,953
Maternal age at delivery, years[a]	29.2±5.7**, †††	32.8±5.5***	28.8±6.0
Duration of diabetes, years[a]	13.2±8.4†††	3.3±3.6	
Parity[a]			
Nulliparous, % (*n*)	50.4 (1604)***, †††	30.5 (438)***	45.9 (368,476)
Multiparous, % (*n*)	49.6 (1585)	69.5 (998)	54.1 (434,672)
SIMD % (*n*)[a]			
SIMD1 most deprived	25.0 (807)‡,§§§	31.0 (449)‡‡‡	25.8 (208,221)
SIMD2	21.1 (680)	22.2 (321)	20.7 (167,327)
SIMD3	20.3 (656)	20.0 (290)	18.6 (149,684)
SIMD4	17.9 (576)	13.7 (198)	17.7 (143,065)
SIMD5 least deprived	15.7 (507)	13.1 (190)	17.2 (138,477)
Maternal smoking in pregnancy % (*n*)[b]	21.5 (635)	21.3 (281)	23.5 (174,749)

Values are presented as mean ± SD or % (n) as indicated.

[a] Excluding 38 pregnancies missing vital status and 249 pregnancies with other maternal diabetes.

[b] Missing data: Maternal age missing in 10 cases, duration of diabetes missing in 56 cases, parity missing in 5861 cases, SIMD missing in 2186 cases, maternal smoking missing in 65,866 cases.

** $p < 0.01$, *** $p < 0.001$ vs no diabetes; †††$p < 0.001$ vs T2DM (Pearson's χ^2 test for categorical variables, ANOVA for continuous variables)

‡ $p < 0.05$, ‡‡‡ $p < 0.001$ vs no diabetes across SIMD classification (Pearson's χ^2 test)

§§§ $p < 0.001$ vs T2DM across SIMD classification (Pearson's χ^2 test)

Figure 3.1: Percentage of total deliveries (born at or beyond 24 weeks gestation) in Scotland with a mother affected by T1DM or T2DM

The solid blue line depicts the percentage of deliveries in Scotland occurring at or beyond 24 weeks of gestation, with a mother affected by pregestational T1DM in each year. The solid red line depicts the percentage of deliveries in the same time period with a mother affected by pregestational T2DM in each year. The corresponding dotted lines show a fitted linear trend, with both T1DM and T2DM demonstrating an increasing prevalence in pregnancy across time ($p<0.001$).

3.3.2 Pregnancy outcomes according to maternal pregestational diabetes status

Table 3.2 shows infant and delivery characteristics. There were marked differences in pregnancy outcomes in women with diabetes compared with the obstetric population without diabetes.

There were 102 perinatal deaths in offspring of mothers with diabetes across the 15 years (64 in T1DM, 38 in T2DM) representing rates 3.3 and 4.3 times those observed in the population without diabetes ($p<0.001$). Stillbirth rates were 3.6-fold higher in T1DM and 5.3-fold higher in T2DM than the non-diabetic population ($p<0.001$) and occurred at a mean gestational age of 33.6 and 34.1

weeks respectively. Stillbirth rates were not improving over time in diabetes (p=0.3 for time trend T1DM, p=0.7 for time trend T2DM as shown in figure 3.2, despite a small decrease in rates in the non-diabetes population (1.4% relative risk reduction per annum). Perinatal mortality was also higher in pregnancies complicated by T2DM at 25.8 per 1,000 births compared with T1DM at 19.6 per 1,000 births (p<0.01), and similarly showed no improvement with time in T1DM or T2DM (Figure 3.3).

Table 3.2: Infant and delivery characteristics according to pregestational maternal diabetes diagnosis

	Type 1 diabetes	Type 2 diabetes	No pre-gestational diabetes[a]
Number of ongoing pregnancies after 24 weeks	3229	1454	808,807
Stillbirths n (*n* per 1000 births)	63 (19.5) ***	36 (24.8)***	3966 (4.9)
Perinatal mortality n (*n* per 1000 births)	65 (20.1) ***	39 (26.9) ***	5154 (6.4)
Multiple pregnancy, % (*n*)	1.2 (40)	1.4 (21)	1.5 (12,166)
Singleton babies % (*n)*	98.8 (3189)	98.6 (1431)	98.5 (796,649)
Mode of delivery [b]			
Elective caesarean section% *(n)*	29.4 (956)***	30.5 (445)***	9.6 (76,776)
Emergency caesarean section % *(n)*	38.3 (1251)***, †††	29.1 (430)***	14.6 (118,284)
Gestation at delivery, weeks[b]	36.7±2.3***, †††	37.3±2.4***	39.3±2.0
Preterm delivery (<37 weeks) % (*n*)[b]	35.3 (1126) ***, †††	21.8 (311)***	6.1 (48,576)
Very preterm delivery (<32 weeks), % (*n*)[b]	3.8 (121)***	3.2 (46) ***	1.1 (8760)
Mean birthweight, g	3466.7±802.8†††	3474.4±793.1***	3398.8±587.9
***Z-score* for birthweight**[b]	1.33±1.34***, †††	0.94±1.34***	0.04±1.01
LGA, % (n)[b]	50.9 (1623)***, †††	38.4 (549) ***	10.5 (84,141)

Values are presented as mean ± SD or *%(n)* unless indicated otherwise.

[a] Excluding 38 pregnancies missing vital status and 249 with other diabetes.

[b] Missing data: mode of delivery missing in 23 cases, birthweight missing in six cases, gestational age at delivery missing in 412 cases.

** $p < 0.01$, *** $p < 0.001$ vs no diabetes; ††† $p < 0.001$ vs T2DM (Pearson's χ^2 test for categorical variables, ANOVA for continuous variables)

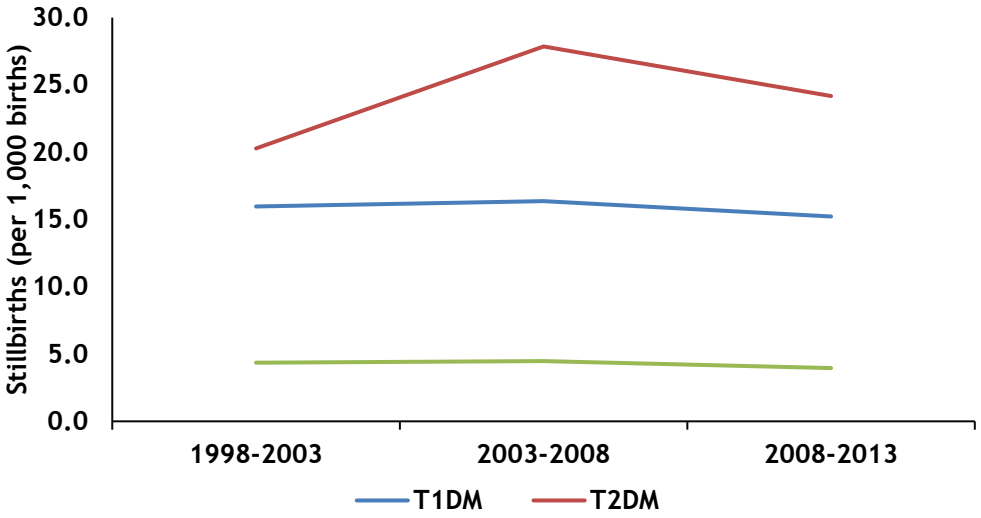


Figure 3.2: Trends in stillbirth rates according to pregestational maternal diabetes diagnosis

Figure 3.2 shows stillbirth rates per 1,000 births according to pregestational diabetes diagnosis. Due to small numbers, rates have been grouped across 5-year periods to improve visualisation of trend data. The blue line denotes mothers with T1DM, the red line mothers with T2DM and those with no pregestational diabetes diagnosis are shown by the green line. Mothers with T2DM have consistently higher stillbirth rates compared with both mothers with T1DM and those mothers without diabetes. Stillbirth rates did not change over time in T1DM (p=0.3 for time trend) or T2DM (p=0.7 for time trend).

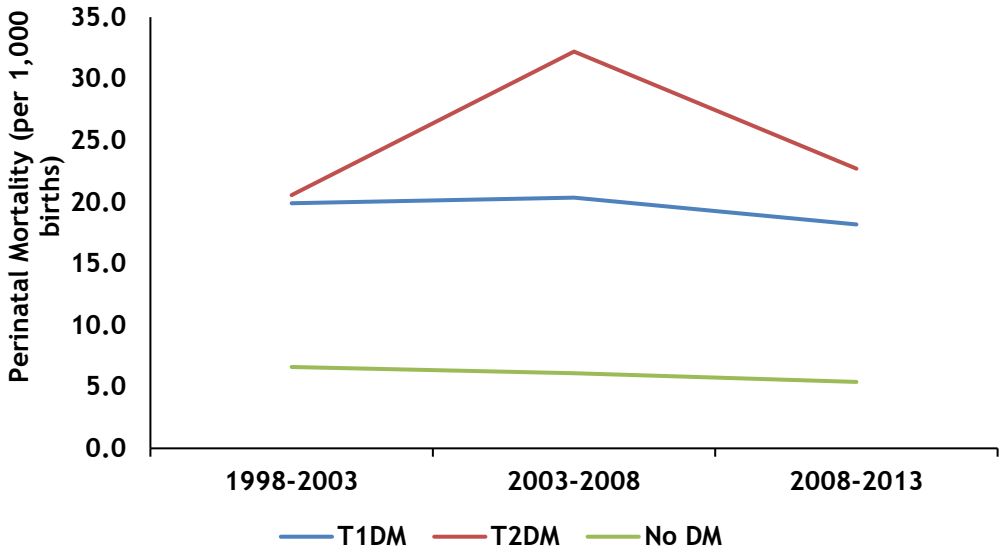


Figure 3.3: Trends in perinatal mortality rates (per 1,000 births) according to pregestational maternal diabetes diagnosis

Trends in perinatal mortality rates are shown across the study time period for mothers with T1DM (blue line), mothers with T2DM (red line) and mothers without pregestational diabetes (green line). As with stillbirth rates, absolute numbers were low and so data is displayed in 5-year time periods for ease of visualisation. Perinatal mortality rates did not have a statistically significant change over time in mothers with T1DM or T2DM, but in the population without pregestational diabetes, there is a 2.2% relative risk reduction per annum (p<0.001).

On average, offspring to mothers with T1DM and T2DM were delivered 2.6 weeks and 2 weeks earlier than in the group without diabetes (Table 3.2). This reflected in higher proportions of preterm (less than 37 weeks' gestation) and very preterm (less than 32 weeks' gestation) deliveries in women with diabetes, with a fivefold increase in preterm delivery in T1DM and almost 4-fold increase in T2DM. The proportions of elective caesarean section (CS) and emergency CS were also greatly increased, with 67.7% of women with T1DM and 59.6% of women with T2DM undergoing operative delivery (Table 3.2)

Despite significantly earlier delivery, mean absolute birthweights in T1DM and T2DM were higher than in those without diabetes (69 g and 76 g heavier in T1DM and T2DM respectively). The absolute difference was only statistically significant in T2DM ($p < 0.001$), however, when adjusted for gestational age, parity and sex, the difference in birthweights were dramatic. Offspring born to mothers with T1DM weighed on average 1.33 SD above those born to mothers without diabetes, and the offspring in T2DM 0.94 SD higher compared to the non-diabetic population. This was reflected in large proportions of LGA infants, with 51% of babies born to mothers with T1DM and 38% born to mothers with T2DM considered LGA (Table 3.2).

3.3.3 Trends in maternal factors and obstetric outcomes across time

3.3.3.1 Trends in maternal factors

Data were then examined for trends across the 15-year time period to assess for any meaningful impact of changing obstetric populations and clinical behaviours that may have occurred. Across the 15 years, mean maternal age at delivery increased by 0.6 years in mothers with T1DM and 1.6 years in mothers with T2DM (Figure 3.4). Maternal age also increased by 0.9 years in mothers without diabetes. The trend to increasing age across time was highly significant ($p < 0.001$) and was similar in mothers with and without diabetes ($p = 0.56$ for time*diagnosis). Mean duration of diabetes also increased, from 12.4 to

14.6 years in T1DM and from 2.6 to 3.8 years in T2DM ($p < 0.001$ for time trend, p=0.11 for time*diagnosis interaction).

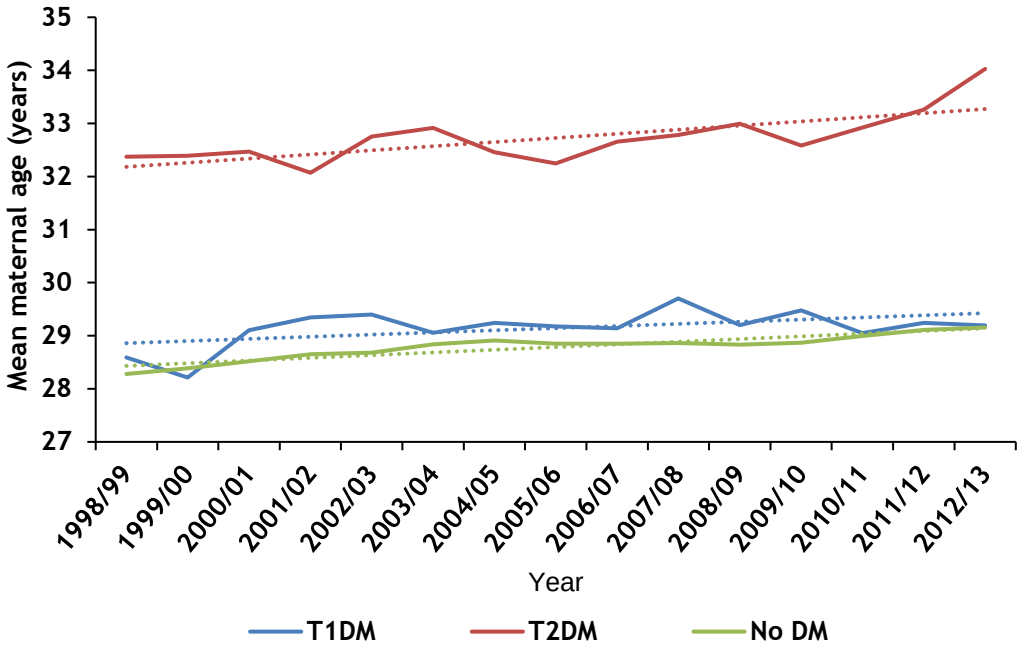


Figure 3.4: Trends in maternal age across time according to diabetes diagnosis

Mean maternal age in T1DM, T2DM and the non-diabetes obstetric populations increased across the audit period ($p<0.001$). Mothers with T2DM, shown by the solid red line, were on average older than mothers with either T1DM (blue line) or no diabetes diagnosis (green line), and this remained consistent across the time period studied. The rate of increase in maternal age across the audit period was similar across all groups, irrespective of diabetes diagnosis (p=0.56 for diagnosis and time interaction terms).

3.3.3.2 Trends in gestational age at delivery and mode of delivery

Women with T1DM and T2DM were delivered at increasingly earlier gestations across the fifteen years. In T1DM, the mean gestational age at delivery fell from 36.7 weeks in 1998/99 to 36.4 weeks in 2012/2013 ($p = 0.03$), whilst in T2DM a similar fall in gestational age at delivery was noted from 38.0 to 37.2 weeks ($p < 0.001$). Figure 3.5 depicts the annual trends in delivery age for women according to pregestational diabetes diagnosis.

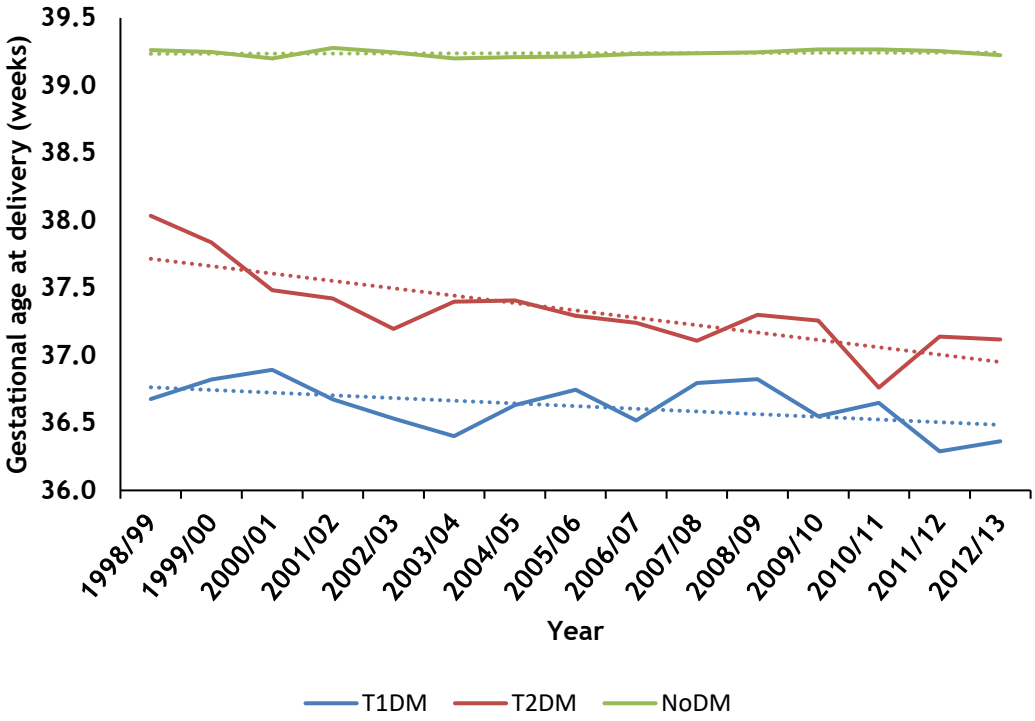


Figure 3.5: Time trends in mean gestational age at delivery according to diabetes diagnosis

Mean gestational age at delivery according to year of delivery and diabetes diagnosis is shown by the solid lines, with a superimposed fitted linear model shown by the corresponding dotted line to allow visualisation of trend data. Mean gestational age at delivery remained constant at >39 weeks for women with no pregestational diabetes diagnosis (shown by the green line), whilst women with both T1DM (blue line) and T2DM (red line) were both delivered increasingly earlier (p=0.03 and <0.001 respectively).

As a reflection of this trend to earlier delivery, the proportion of women delivering singleton infants preterm (before 37 weeks gestation) increased for both groups, from 34.1% to 42.4% for T1DM (p=0.001), and a dramatic increase from 11.9% to 25.5% in T2DM (p=0.003). Very preterm deliveries (under 32 weeks) were uncommon (Table 1) and showed no significant trends.

The proportion of singleton deliveries involving CS also increased across all groups (p<0.001 for both elective and emergency CS) as shown in figure 3.6 For the obstetric population without diabetes, there was a significant increase from 6.8% to 11.8% for elective CS and from 11.8% to 15.1% for emergency CS. Elective and emergency CS rates were far higher in mothers with T1DM and T2DM (p < 0.001 for both compared to those without diabetes), yet still showed the same upward trajectory. The only exception was emergency CS in women

with T1DM for which the already very high rates (40%) remained stable over time.

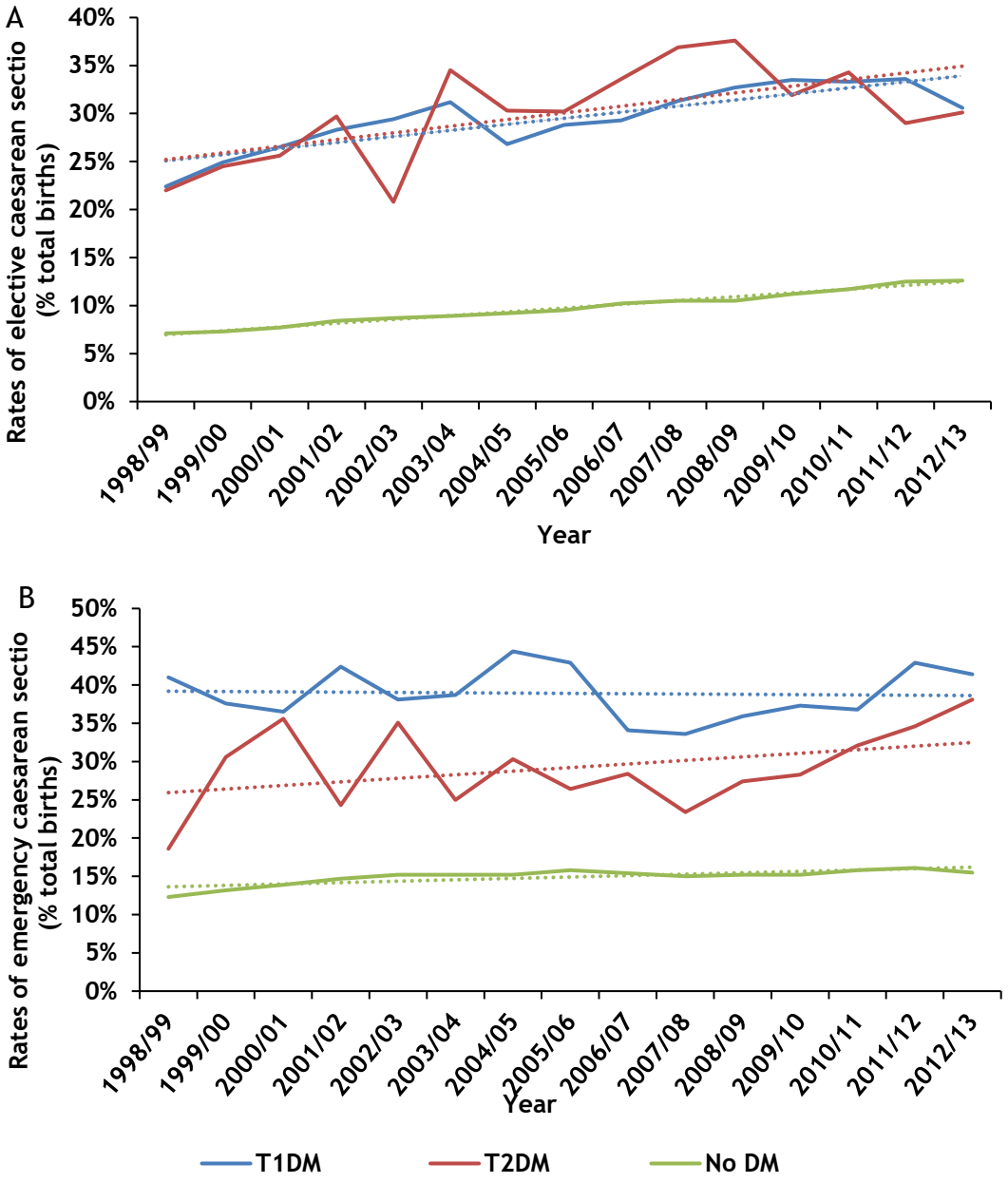


Figure 3.6: Time trends in rates of elective caesarean section (Fig A) and emergency caesarean section (Fig B) according to diabetes diagnosis.

Rates of elective CS increased with time in T1DM (blue line), T2DM (red line) and the non-diabetes obstetric population (green line) as shown in figure A ($p<0.001$ for time). The solid lines denote the rate of CS at each time point, whilst the corresponding dotted lines show a fitted linear trend. Women with T1DM and T2DM had significantly increased baseline rates of Elective CS compared with mothers without diabetes, but despite this higher baseline continued on a similar increasing trajectory ($p=0.15$ for time*diagnosis interaction). Emergency CS rates are shown in panel B. Women with T1DM had the highest rates of emergency CS and remained stable at this rate over time, which is in contrast to the increasing rates seen in T2DM and women without diabetes ($p<0.001$).

3.3.3.3 Trends in birthweight outcomes

Figure 3.7 shows changes in mean birthweight Z-scores (adjusted for gender, gestational age and parity) with time, according to diabetes diagnosis. Across the audit period, mean birthweight Z-scores appeared stable in offspring of women with T2DM (p=0.9 for time trend), but increased across time in T1DM from 1.22 to 1.47 SD above the reference population (p =0.001 for time trend). This was reflected in the proportion of LGA infants increasing from 47.1% to 56.2% in T1DM, and static rates in T2DM as shown in figure 3.8.

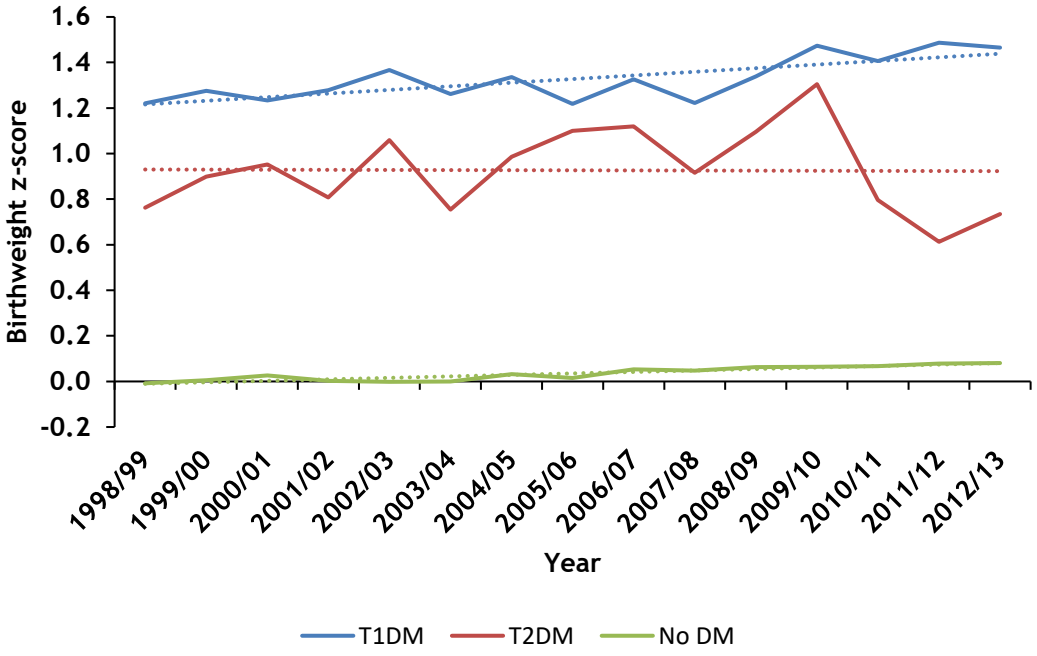


Figure 3.7: Trends in mean infant birthweight Z-scores across time according to maternal diabetes diagnosis

Mean infant z-birthweight scores adjusted for gestational age, parity and infant sex are shown here, with the reference population data derived from published data on all Scottish births between the years 1998-2003 (Bonellie et al., 2008). In both infants born to mothers with T1DM and to those mothers without diabetes, the mean birthweight Z-score increased with time (p=0.001 for time trend in T1DM and no DM groups), whilst in T2DM, it remained stable albeit significantly higher than the population without diabetes (p<0.001 for T2DM versus no DM).

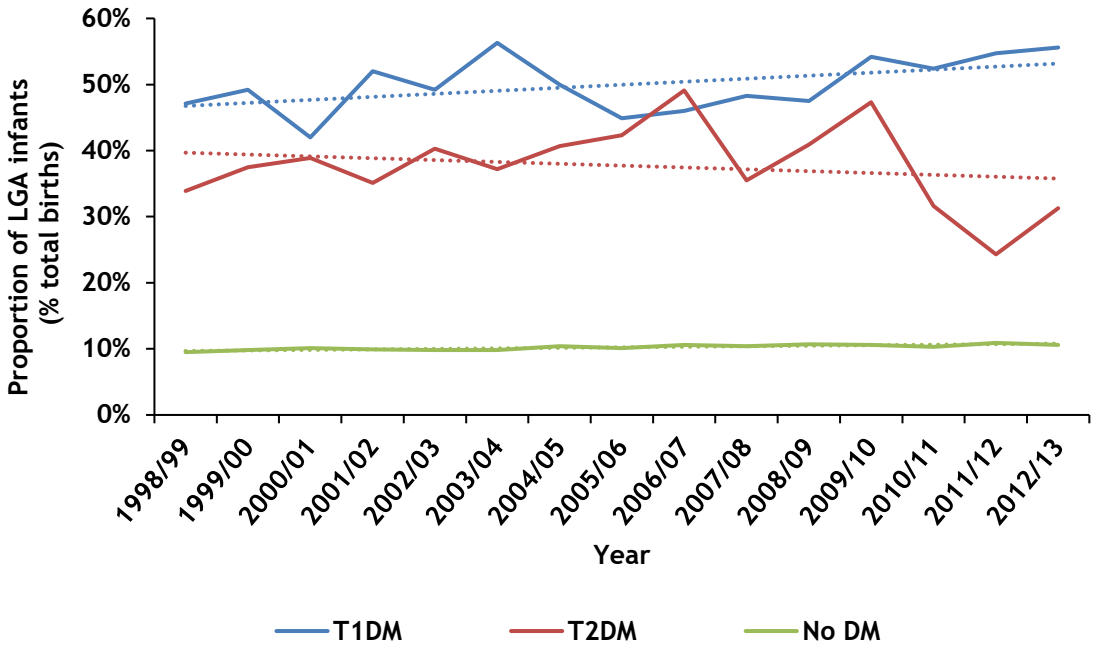


Figure 3.8: Time trends in proportion of infants born LGA (>90th centile birthweight adjusted for gestational age, gender and parity) according to diabetes diagnosis

This figure shows the rates of LGA infants born in each category of maternal diabetes status. The solid lines depict the absolute rates, as a percentage of total births within that category whilst the dotted line shows a fitted linear trend. In keeping with the definition of LGA as an adjusted birthweight > 90th centile, infants born to mothers without diabetes have an approximate 10% incidence of LGA birthweights. This appeared stable over time. LGA rates were much higher in the population with T1DM and T2DM, at approximately 50% and 40% of births affected. LGA rates increased significantly with time in the type 1 diabetes population ($p<0.001$), but did not change significantly in T2DM.

Maternal smoking status was analysed for an association with birthweight. Smoking rates reduced significantly across the fifteen-year period from examined from 24.1% to 18% in T1DM, 22% to 14% in T2DM, and 27.9% to 19.3% in mothers without diabetes ($p < 0.0001$ for time trend with no difference between groups $p=0.7$). In a model that included time, maternal smoking status and SIMD deprivation scores, smoking was associated with lower birthweight in both T1DM ($\beta=-0.181$, $p < 0.001$) and T2DM ($\beta=-0.083$, $p = 0.003$). Social deprivation (as represented by the SIMD) did not have an effect on birthweight. The trend to increased birthweight Z-score T1DM over time was attenuated by the addition of smoking as a variable in the model, but remained significant ($p=0.017$).

3.4 Discussion

Birthweight, prematurity, operative delivery and perinatal mortality represent key outcome measures in the management of pregnancy complicated by diabetes. The results of this study therefore raise concern for the clinical teams involved with managing these women in Scotland. Specifically, already high stillbirth and perinatal mortality rates have remained stable over time, and birthweight is also increasing in T1DM. Higher rates of operative delivery and falling gestational age at delivery suggest increasing obstetric intervention, likely as a preventative measure to reduce such outcomes but granular data on their impact are lacking.

In Scotland, stillbirth rates in T1DM appear intermediate between 2002/2003 and more recent data from 2013 and 2018 in England and Wales (25.8 per 1000 births to 10.3-10.7 per 1000 births) (CEMACH, 2005, National Diabetes in Pregnancy Audit Report 2018, 2019, National Diabetes in Pregnancy Audit Report 2013, 2014). However, there is wide variation across European nations who each use differing data capture methodologies. In T1DM, Scottish stillbirth rates are similar to slightly older Swedish data showing 15 stillbirths per 1000 deliveries (1991–2003), but is lower than in the French or Dutch surveys (Persson et al., 2009, Diabetes and Pregnancy Group France, 2003). Thankfully, absolute stillbirth numbers are low, but this limits the power of such surveys to detect meaningful trends in mortality outcomes. As such, direct comparison between nations is challenging. Although stillbirth rates in T1DM in Scotland do not appear formally significantly different from England and Wales data, there is concern that we are not on a similar improving trajectory. This is especially true in T2DM, where the Scottish stillbirth rate is both significantly higher than seen in the Scottish T1DM and that observed in the 2018 England and Wales survey (16.7 per 1,000 births) (National Diabetes in Pregnancy Audit Report 2018, 2019). Notably, any difference appears to relate to stillbirth rather than neonatal mortality (death during the first 28 days of life), which is not changing greatly in England and Wales (10.4 per 1000 livebirths for all types of diabetes) and is highly comparable to Scottish perinatal mortality rates (National Diabetes

in Pregnancy Audit Report 2018, 2019, CEMACH, 2005). However, there are major caveats to direct comparison of Scottish rates with these data, not least due to differences in data collection methodologies (patient opt-out and clinical centre opt-in of data provision permitted in England and Wales versus whole-population inclusion in Scotland). The population of Scotland is also much smaller resulting in similar numbers of patients included in our analysis, but with data collected across a much wider range of years. However, epidemiological data also suggest that the Scottish population experiences a higher burden of cardiometabolic disease than those in England (Scarborough et al., 2011), and on a whole population level have higher rates of obesity and smoking (Bromley and Shelton, 2010). Whilst these data do not pertain specifically to the obstetric population, if similar phenotypes were evident in this group then it could contribute additional stillbirth risk.

Operative delivery rates also appear high in Scotland (67.7% for T1DM, 59.6% for T2DM) and are higher than equivalent data from France, Sweden and the Netherlands (44.3–58.9%) (Evers et al., 2004, Persson et al., 2009, Diabetes and Pregnancy Group France, 2003). They are however similar when compared to England and Wales which has CS rates of 66% for T1DM and 56% for T2DM (National Diabetes and Pregnancy Audit Report, 2014). The rates of elective CS in T1DM and T2DM, and most strikingly emergency CS in T2DM, are also increasing over time. Although the proportions of emergency CS in T2DM (29%) appear very similar to those from England and Wales, this compares with a figure of just 10% in the Netherlands (de Valk et al., 2006), perhaps reflecting different obstetric behaviours between mainland UK regions and other similar developed nations.

A potential driver for increasing obstetric intervention is the perceived risk of increasing birthweight and accelerated fetal growth often seen in pregnancy complicated by hyperglycaemia (Pedersen, 1952). Average birthweight is greatly increased in the offspring of mothers with T1DM and T2DM in Scotland - despite the best efforts of individuals and their clinicians to optimise glycaemia. These data reinforce the already acknowledged clinical need to improve upon

glycaemia in pregnancy. This is particularly true in women with T1DM where glycaemic excursions can be more frequent and more difficult to manage. In Scotland, the 'average' baby born to a mother with T1DM in Scotland has a birthweight just above the 90th percentile and half of them are born LGA, which is marginally higher than LGA rates in T1DM in England and Wales in 2013 (46.4%). Rates of LGA infants in mothers with T2DM were also higher during the same time period (38% vs 24%) (National Diabetes and Pregnancy Audit Report 2013, 2014). There will be important confounding factors other than diabetes and glycemia influencing birthweight that have not been measured due to limitations in the dataset. For example, ethnicity will have become more diverse over the time period, particularly for T2DM. However, census data confirms a largely (>96%) white population, and so any effect from this is likely to be moderate at best (Census 2011 (National Records of Scotland), 2013). Maternal BMI is considered one of the strongest predictors of infant birthweight (independent of maternal glycaemia). Whilst some pre-pregnancy BMI data could be obtained from SCI-Diabetes for women with T1DM or T2DM, the data for the comparator non-diabetes group on SMR02 are less reliable (Assessment of maternity data (SMR02) 2008-09 (Information Services Division Scotland), 2010). Customised growth centiles are used in England and Wales (adjusted for maternal height, weight and ethnicity), whilst the Scottish datasets utilises centiles adjusted for gestational age parity and sex based on a very large number (>100,000) of contemporaneous Scottish births. Debate remains about which is the best predictor of adverse perinatal outcome. In one study, partially customised centiles applied to the Scottish obstetric populations did not appear to show any discriminatory difference in detecting neonatal mortality or stillbirth risk. However this study had a caveat that weight and ethnicity data were lacking (Iliodromiti et al., 2017). Recent analysis of CONCEPPT trial data showed that in a T1DM population with high LGA rates, customised GROW centiles which take into account maternal height, weight and ethnicity showed a better association with neonatal hypoglycaemia and need for NICU versus population-standardised centiles (Meek et al., 2021).

In this study, a number of observed trends were expected. Although diabetes in pregnancy remains relatively uncommon (1 in 178 births in my data), the prevalence of both T1DM and T2DM complicating pregnancy is increasing with time. Although somewhat speculative, reasons for this increase may reflect higher rates of maternal obesity, advancing maternal age and small increases in the size of at-risk ethnic groups seen in the general Scottish population (Waugh et al., 2011, Census 2011 (National Records of Scotland), 2013). The prevalence of diabetes in the general population is also increasing over these years (Scottish Diabetes Survey Monitoring Group, 2014). Whilst this group represent a small proportion of the overall obstetric population, there are important health resource and spending implications from increasing numbers needing seen in specialist clinics. The increase in duration of both T1DM and T2DM is also of importance, as this may translate into increased rates of microangiopathy with associated risks of placental insufficiency, IUGR, preeclampsia and stillbirth (Ekbom et al., 2001).

Some of the trends found were less expected. Mothers with T1DM and T2DM are being delivered increasingly earlier and caesarean section rates are increasing but, despite this, adjusted birthweight is on the rise. In contrast, the population without diabetes appear to have a stable gestational age at delivery and birthweight increases are much smaller over time (<0.1 SD across 15 years). Similar to the population with diabetes, rates of elective and emergency CS are increasing significantly with time. I would be interested in the clinical reasoning for choosing CS, but the datasets lack this level of detail. Similar rising trends in elective CS rates across groups suggest that general obstetric practice might be changing, but this does not explain the rising emergency CS rates in T2DM. In contrast to the Scottish population, it is noticeable that in England and Wales, emergency CS rates in women with diabetes are decreasing (37.6% in 2003/2004, 30% in 2013) (CEMACH, 2005, National Diabetes in Pregnancy Audit Report 2013, 2014). This might reflect subtle changes in clinical guidelines where delivery is expected to occur sometime between 37+0 and 38+6 weeks of gestation in England and Wales, whilst Scottish guidelines recommend counselling otherwise well women with diabetes for delivery at or shortly after 38 weeks, and certainly

before 40 weeks gestation (Management of Diabetes (Scottish Intercollegiate Guidelines Network), 2017, Diabetes in Pregnancy (National Institute of Health and Care Excellence), 2015). Alternatively, the increase in emergency CS may reflect a more adverse metabolic phenotype of the Scottish obstetric population with associated increased obstetric risk, factors which are not easily measured using these routine clinical data.

Increasing LGA rates in T1DM appear similar to other UK nations (National Diabetes in Pregnancy Audit Report 2018, 2019). Other nations have also reported increasing trends. In Sweden, the proportion of LGA babies was reported as 23%, 31% and 47% in successive reports of cohorts in their populations of 1982–1985, 1991–2003 and 1998–2007 (Metzger et al., 2010, Persson et al., 2009, Persson et al., 2011). Interestingly, this was not attributed to deteriorating glycaemic control, which was probably becoming tighter. Data on glycaemic control in my study were not standardised across the whole time period as all laboratories became DCCT-aligned at differing points pre-2006. This makes temporal assessment of glycaemic control difficult but anticipate that a deterioration would have been unlikely, as this was not observed in the previous surveys conducted in 1998/1999 and 2003/2004 (Kernaghan et al., 2006, Penney et al., 2003). Smoking is also known to cause a reduction in birthweight, and thankfully has reduced significantly in pregnancy in our population over the time course of the study. Accounting for this may provide a very minor contribution to the rise in z-scores in T1DM, but is clearly not a health behaviour to be recommended to pregnant mothers with diabetes. On the other hand, social deprivation as measured by the gross SIMD score, did not appear to influence birthweight in women with T1DM or T2DM. It is important to understand these health and social factors that may influence LGA, since LGA carries an increased risk of adverse perinatal outcomes (Persson et al., 2012).

The main strengths of this study are its large scale and inclusivity of all pregnancies in Scotland, thereby avoiding selection bias. These routinely collected clinical data used are subject to regular quality assurance checks, and proven to be of both high quality and accuracy. Registration of individuals into

SCI-Diabetes occurred in the various Scottish health boards between 2003 and 2006, including an upload of previously held electronic clinical and biochemical records on current patients in those years. Women who delivered prior to 2003 and subsequently left Scotland may not have had their data entered on SCI-Diabetes and thus may have not been included in this study. This is likely to represent very few women. The data used in this study appear robust when compared with previous national paper audits. Only a slightly higher number of deliveries were ascertained in 2003/2004 than from the previous paper audit for that time period (172 vs 165 deliveries respectively) (Kernaghan et al., 2006), and 96% of deliveries ascertained compared with the 1998/1999 paper audit (Penney et al., 2003). It would appear safe to assume that the population of women with diabetes is relatively stable, and that ascertainment is successful. The development of similar national audits, most notably in England and Wales, which included 86% of consultant-led obstetric units in 2013, is welcome as it allows meaningful comparisons of outcomes between countries (National Diabetes in Pregnancy Audit Report 2013, 2014). This allows us the opportunity to learn from different approaches and models of care.

Results from the previous Scottish surveys reported a higher Z-score in T1DM (1.64 in 2003/2004 in the previous paper survey compared with 1.34 for that year in my data). All data are provided in a pseudonymised format with restrictions on reporting case numbers where n is less than 5. This is to protect patient anonymity and confidentiality but also means I cannot directly compare inclusion in the previous surveys case by case. The difference in Z-score between the previously published data and mine is likely to reflect the standard used. The previous national surveys used reference data on births from a single centre in Aberdeen between 1979–1983, whereas the present study used a contemporaneous, whole Scottish population standard (Kernaghan et al., 2006).

Pseudonymised data also limit any retrospective identification and analysis of case records, and so any data inclusion is restricted to information electronically captured within the SMR02 and SCI-Diabetes datasets. However, it is major strength to use these routine clinical records in that it avoids under-reporting of

adverse outcomes by individual units, and the reporting of outcomes such as weight, delivery method and gestational age is known to be robust. However, it does not allow measurement of aspects of care that have previously been assessed in paper surveys. Factors such as attendance at prepregnancy clinics are not recorded, detailed information on congenital anomalies and important perinatal outcomes such as neonatal intensive care admissions are lacking. Prenatal glycaemic control has previously been shown to be unchanged between 1998/1999 and 2002/2003 and is suboptimal, with only 54% of women having documented preconceptual HbA_{1c} values, and with average levels 30% above normal (Kernaghan et al., 2006). It seems likely that preconceptual preparation remained suboptimal in this cohort across the study period. Data from England and Wales suggest only 1 in 8 women with pregestational diabetes have optimised diabetes care preconceptually (National Diabetes in Pregnancy Audit Report 2018, 2019). Inadequate preparation for pregnancy may lead to higher rates of congenital malformation and pregnancy loss. It would also be of interest to be able to account for other maternal factors such as gestational weight gain, which may impact infant growth and placental function. Reliable data on hypertensive disorders of pregnancy would also be of interest.

These data have shown that pregnancy for women with diabetes remains high risk, and much is still to be understood regarding causes and effective interventions for adverse outcomes. As has been shown for other aspects of diabetes care (Mcknight et al., 2015), comparison of rates of complications across different countries and healthcare systems offers an important opportunity to understand the potential for improvement both in Scotland, and on a wider international level. This includes understanding ways to improve pre-pregnancy care, defining and achieving optimal glycaemic control during pregnancy and sharing knowledge of good obstetric practice to reduce risk. Further exploration of specific risk factors and their contribution to adverse pregnancy outcomes in diabetes is warranted before meaningful policy development can occur.

4 Chapter 4: Stillbirth and associated risk factors in pregnancy complicated by diabetes

The work within this chapter has been published in Diabetologia, Oct 2019. 61(10):1938-1947 (Mackin et al., 2019)

4.1 Introduction

I have shown in the last chapter, mothers with pregestational diabetes are at 4-5-fold increased risk of stillbirth, and that rates are not improving with time in Scotland (Mackin et al., 2018). This data compares similarly to some other populations where stillbirth rates in diabetes remain static in recent years (Feig et al., 2014), but contrasts to the decreasing stillbirth rates seen in the Scottish obstetric population without diabetes. Maternal obesity, advanced maternal age and smoking are recognised as important modifiable risk factors for stillbirth in the general obstetric population (Gardosi et al., 2013, Flenady et al., 2011). Fetal growth is also important, with growth-restricted pregnancies having the highest risk (Gardosi et al., 2013). This knowledge comes from large studies in the general obstetric population but studies specific to risk of stillbirth in diabetes are more limited. Suboptimal maternal blood glucose levels even at minimal levels, presence of microvascular complications and poor preparation for pregnancy are all associated with stillbirth (Lauenborg et al., 2003, Tennant et al., 2014, Murphy et al., 2021). Other traditional risk factors seen in the general obstetric population are less well documented in the population with diabetes. Prevention of stillbirth underpins part of the clinical rationale for increased obstetric intervention in diabetes, particularly around timing of delivery. However, there is a distinct lack of good predictive models to identify impending stillbirth risk, and so obstetricians are often required to assess presence of known risk factors to justify earlier delivery in women. In many cases, earlier delivery is appropriate but the benefits can be negated by increased risks of neonatal morbidity as a result of premature delivery (Stutchfield et al., 2005). The aim of this study was to define the maternal and fetal characteristics associated with stillbirth in pregnancy complicated by

diabetes, including regional variation in outcomes and timing of delivery in such pregnancies. This would allow us a better understanding of the pregnancy phenotype at risk and inform discussion around improved health strategies to better detect such pregnancies and offer them appropriate intervention.

4.2 Methods

Maternal and delivery characteristics in pregnancies complicated by diabetes that ended in stillbirth were compared with those who ended in livebirth. Similar to chapter 3 methodology, data from maternity records in SMR02 database were linked to the national diabetes database SCI-Diabetes and is described in detail in chapter 2.1.

Episodes that resulted in the delivery of an infant at or beyond 24 weeks of gestation to mothers with pre-existing T1DM or T2DM from 1 April 1998 to 30 June 2016 were identified. Analysis was restricted to singleton births only.

4.2.1 Statistical analysis

Crude stillbirth rates are presented and when analysed for any given gestational age they are presented with a denominator of continuing pregnancies at that particular gestation.

Risk factors and pregnancy outcomes are presented according to diabetes diagnosis for those in stillbirth and livebirth groups as means and SDs, or frequencies, as appropriate. Comparison of risk factors and outcomes between groups was assessed using a generalised mixed model with a term for the mother incorporated as a random effect.

Differences in outcomes between health board and hospital unit size were analysed using logistic regression or general linear model with additional terms for year of delivery, deprivation score, maternal age, smoking and diabetes duration as appropriate. Due to low absolute numbers, effect of health board area of delivery and unit size on stillbirth rates was calculated from data

combining T1DM and T2DM deliveries together but including a term for type of diabetes in the model.

4.3 Results

There were 5621 pregnancies in mothers with diabetes, of which 229 were excluded for reasons including missing gestational age at delivery ($n = 7$), missing sex of infant ($n = 3$), delivery before 24 weeks of gestation ($n = 5$), diabetes diagnosis other than T1DM or T2DM ($n = 145$) or twin pregnancy ($n = 74$). Exclusions could have been in more than one category.

The remaining singleton babies included 3778 infants born to 2582 mothers with T1DM and 1614 infants born to 1265 mothers with T2DM. Unsurprisingly stillbirth rates were similar to those in the previous study at 16.1 per 1000 births (95% CI 12.4, 20.8) in T1DM ($n = 61$) and 22.9 per 1000 births (95% CI 16.4, 31.8) in T2DM ($n = 37$).

4.3.1 Type 1 diabetes

Table 4.1 shows maternal and infant characteristics for pregnancies complicated by T1DM according to live and stillbirth outcomes. Mean maternal age, parity, maternal smoking rates and deprivation scores were similar in mothers regardless of whether the pregnancy ended in stillbirth or livebirth. Mothers who experienced a stillbirth had a slightly lower BMI and lower duration of diabetes ($p<0.05$ for both) than those who delivered a livebirth.

Table 4.1: Maternal and infant characteristics in mothers with T1DM according to vital status of infant

	Type 1 diabetes (n=3778 babies to 2582 mothers)	
	Stillbirth (n=61)	**Livebirth (n=3717)**
Maternal age at delivery, years (SD)	29.1 (5.3)	29.8 (5.7)
SIMD score (SD)	25.4 (16.4)	23.5 (17.1)
Duration of diabetes, years (SD)	11.4 (9.2)* †	14.1 (8.4)
Nulliparous	55.7%	50.0%
Maternal Smoking	25.5%	20.4%
Pre-pregnancy maternal BMI (SD)	24.8 (4.9) * †	26.3 (4.9)
% Preconception HbA1c below 53 mmol/mol (7%) (n)[a]	7.9%* (3 of 38)	20.0% (438 of 2236)
Male fetus	54.1%	49.4%
Gestational age delivery, weeks (SD)	33.8 (4.1) ***	36.6 (2.2)
Birth weight Z-score (SD)	1.38 (1.69)	1.37 (1.30)

Values are presented as % of group, or mean ± SD.

*p<0.05, **p<0.01, ***p<0.001 for stillbirth versus livebirth by, χ^2 or t-test as appropriate. ([a] by Fishers exact test)

† p<0.05 in general linear model for stillbirth versus livebirth

Missing values (n): Parity: 40; Maternal smoking: 294; Pre-pregnancy BMI: 1302; preconception HbA1c: 1427; birth weight Z-score: 50.

4.3.1.1 Glycaemia in mothers with type 1 diabetes

HbA1c measurements in each trimester and the pre-pregnancy stages were available in SCI-diabetes for 50-60% of cases. Stillbirth rates were no different in mothers who had a prepregnancy HbA1c measured compared to those without a measure (1.6% in both groups, p = 0.9). This was despite higher deprivation scores (25.0±17.6 versus 22.6±16.7, p<0.001) and higher maternal smoking rates (26.1% versus 17.0%) in those without a pre-pregnancy HbA1c measure.

One in five women achieved pre-pregnancy glycaemic targets of <53 mmol/mol. Notably, there was a lower number of women achieving this target in the stillbirth group compared with the livebirth group (7.9% versus 20.0%, p<0.05). I then went on further to examine temporal trends in HbA1c across pregnancy and to compare the live and stillbirth groups. Women who experienced a stillbirth had higher mean pre-pregnancy HbA1c (11 mmol/mol higher) than those pregnancies resulting in a livebirth (p = 0.0002). Whilst both groups improved

their HbA1c across successive trimesters, mothers who had a stillborn infant maintained a similar level of hyperglycaemia relative to their liveborn group across each stage of pregnancy. Figure 4.1 demonstrates mean HbA1c for each stage.

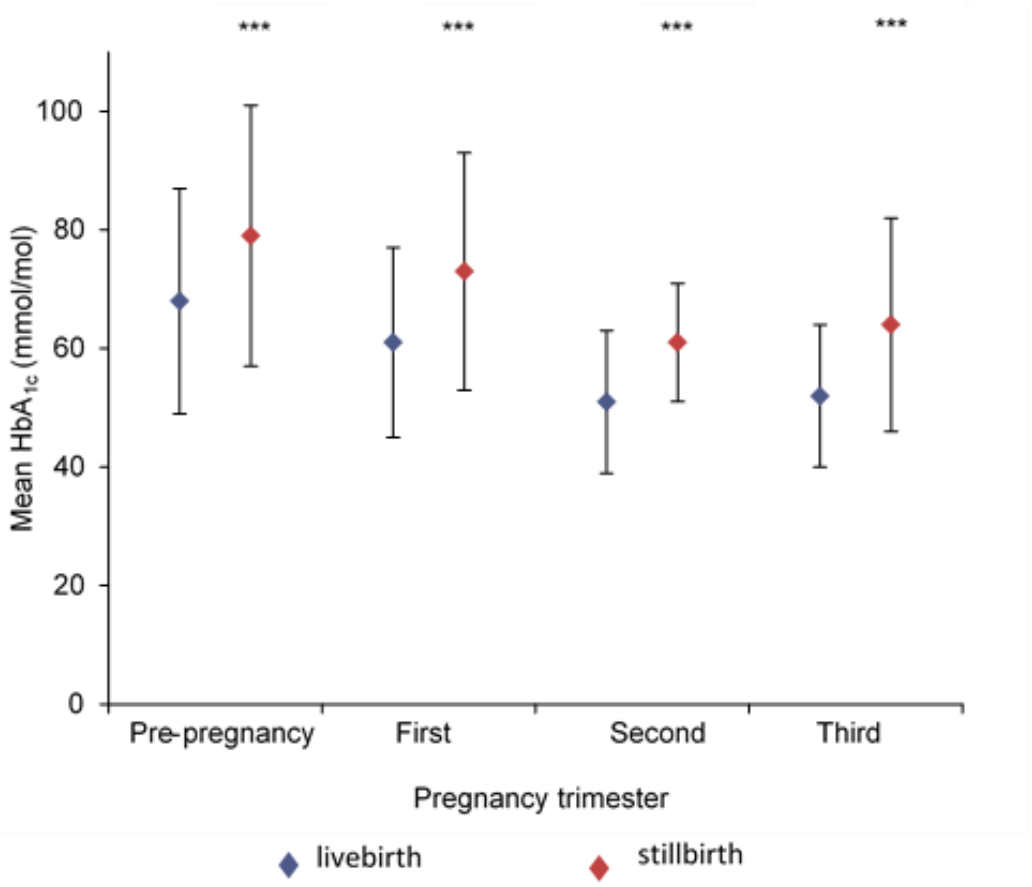


Figure 4.1: Mean HbA1c in mothers with T1DM at each stage of pregnancy according to infant vital status at birth

Mean maternal HbA1c at each stage of pregnancy is demonstrated for the mothers who delivered a liveborn infant (blue diamond) and the mothers who delivered a stillborn infant (red diamond). Error bars demonstrate the corresponding SD.

***p<0.001 for stillbirth versus livebirth for that pregnancy stage by t-test

Number of available HbA1c values:

pre-pregnancy: 2313 (62%) livebirths and 38 (63%) stillbirths; 1st trimester: 2156 (58%) livebirths and 30 (49%) stillbirths; 2nd trimester: 2119 (57%) livebirths and 29 (48%) stillbirths; 3rd trimester: 1933 (52%) livebirths and 27 (44%) stillbirths

Mothers who experienced a stillbirth had consistently higher mean HbA1c values from pre-pregnancy and spanning each trimester compared to the livebirth group. Importantly, neither group of women managed to achieve a normal HbA1c (<48 mmol/mol) and only the livebirth group managed to achieve <53 mmol/mol during pregnancy (in the second and third trimesters).

4.3.1.2 Type 1 diabetes and timing of delivery in relation to stillbirth

In the T1DM group, stillborn infants were born 2.8 weeks earlier than liveborn infants (as shown in table 4.1). Timing of delivery of a stillborn infant was used a proxy for estimating gestational age at which stillbirth occurred. Stillbirths occurred across a wide range of gestations between 24 and 38 weeks. Almost 40% of stillbirths were delivered at or beyond 37 weeks as shown in figure 4.2, whilst 63% of livebirths were delivered at term.

When expressed specific to each week of pregnancy, stillbirth rates in T1DM peaked in the 37th and 38th weeks at 5.1 (95% CI 2.8, 9.1) and 7.0 (95% CI 3.7, 12.9) per 1,000 ongoing pregnancies at that gestation respectively. Figure 4.2 shows stillbirth rates according to gestational age, with corresponding numbers of livebirths for that particular gestation. There were no stillbirths after 38 weeks, and only 11% of all deliveries occurred beyond this likely reflecting adherence and some success of national policy on delivery timing.

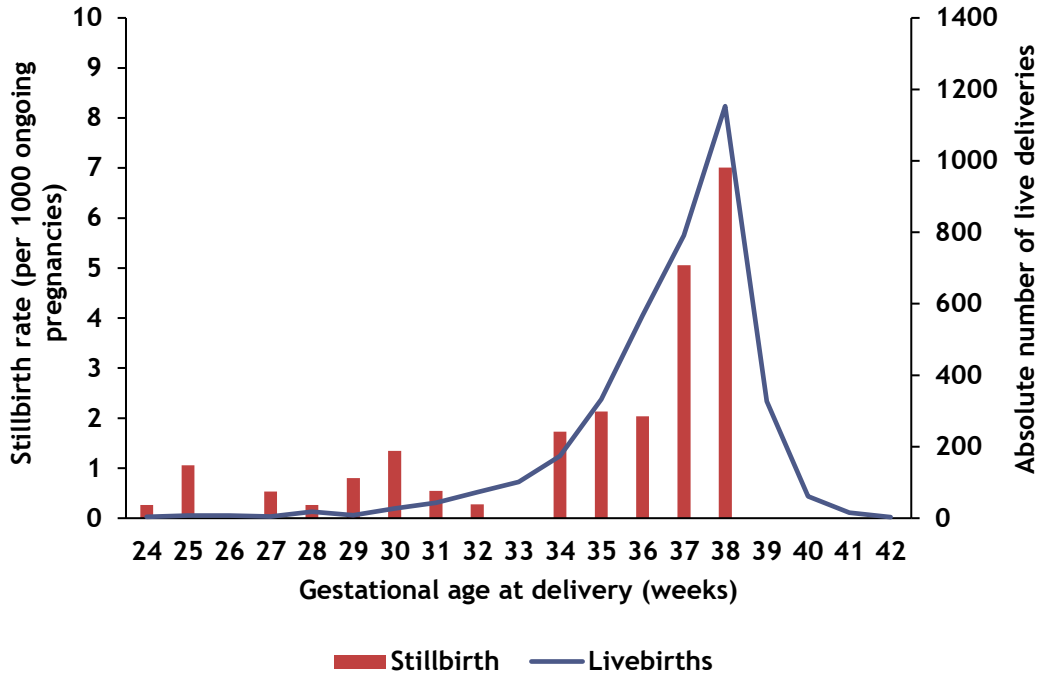


Figure 4.2: Stillbirth rate (per 1,000 ongoing pregnancies) and corresponding number of liveborn deliveries according to gestational week of delivery in T1DM

This figure shows the stillbirth rate at each gestational week expressed per 1,000 ongoing pregnancies for that particular week of gestation (x-axis) in mothers with T1DM. The red bars show the crude stillbirth rate for that gestational week with the primary y-axis (left) quantifying the rate. The blue line demonstrates the absolute number of liveborn deliveries occurring at each gestational week, for which the secondary y axis (right) is used for quantification.

4.3.1.3 Infant birthweight and stillbirth risk in pregnancy complicated by type 1 diabetes

As demonstrated in table 4.1, stillborn infants had similar corrected birthweights to liveborn infants (birthweight Z-scores 1.38 and 1.37 respectively despite earlier gestation at delivery. Table 4.2 categorises infants according to birthweight centile categories. Half of infants (52% combined live and stillborn) had birthweights in the LGA category, and of these, the vast majority (78%) were above the 95th centile.

When compared against appropriate for gestational age infants (10-90th centile), there was a significant effect of birthweight centile category on stillbirth risk, particularly in those born small for gestational age (<10th centile) who had a 6-fold higher prevalence of stillbirth (p=0.005).

Table 4.2: Stillbirth prevalence according to birthweight centile category in infants born to mothers with T1DM

	Birthweight centile (total n within category)			
	<10th (n=72)	**10th-90th (n=1705)**	**>90th (n=1951)**	**>95th (n=1527)**
% stillbirth (n)	6.9% (5)	1.2% (20)	1.7% (34)	1.7% (26)
% livebirth (n)	93.1% (67)	98.8% (1685)	98.3% (1917)	98.3% (1501)
Odds ratio (95% CI)	6.3 (2.3-17.3)	REF	1.5 (0.9-2.6)	1.5 (0.8-2.6)

Missing birthweight values: n=50

4.3.2 Type 2 diabetes

Table 4.3 shows maternal and infant characteristics for those pregnancies complicated by T2DM. Overall, the average BMI in mothers with T2DM fell within the obese range (>30kg/m^2). For mothers in the stillbirth group, mean pre-pregnancy BMI was significantly higher than those mothers who delivered live infants (38.2$\pm$6.4 versus 33.2$\pm$5.6 respectively, p=0.01). The stillborn group had lower rates of primiparous mothers compared with liveborn (16.7% versus 31.3%) but this did not reach statistical significance. There were no significant differences in any of the other measured maternal demographics.

Interestingly, there was a significantly higher rate of male infants in the stillborn group compared with the liveborn group (p = 0.0007).

Table 4.3: Maternal and infant characteristics in mothers with T2DM according to vital status of infant

	Type 2 diabetes (n=1614 babies to 1265 mothers)	
	Stillbirth (n=37)	**Livebirth (n=1577)**
Maternal age at delivery, years (SD)	33.8 (6.0)	33.2 (5.6)
SIMD score (SD)	28.4 (19.1)	28.0 (18.1)
Duration of diabetes, years (SD)	4.4 (4.0)	4.2 (4.1)
Nulliparous	16.7%	31.3%
Maternal Smoking	30.3%	21.0%
Pre-pregnancy maternal BMI (SD)	38.2 (6.4)*†	33.9 (7.1)
% Preconception HbA1c below 53 mmol/mol (7%) (n)[a]	23.5% (4 of 14)	47.6% (47.6)
Male fetus	81.1%***	50.5%
Gestational age delivery, weeks (SD)	33.7 (4.7) ***	37.2 (2.3)
Birth weight Z-score (SD)	1.08 (1.8)	0.8 (1.4)

Values are presented as % of group, or mean $\pm$ SD.

$^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ for stillbirth versus livebirth by, χ^2 or t-test as appropriate. ([a] by Fishers exact test)

† $p<0.05$ in general linear model for stillbirth versus livebirth

Missing values (n): Parity: 13; Maternal smoking: 128; Pre-pregnancy BMI: 776; preconception HbA1c: 801; birth weight Z-score: 14.

4.3.2.1 Glycaemia in mothers with type 2 diabetes

Similar to the population with T2DM, HbA1c measurements were only available for a proportion of the population. Approximately 50% of mothers in the pre-pregnancy stage and in each trimester had an available HbA1c measure. Again, the stillbirth rates were similar regardless of whether women had a pre-pregnancy HbA1c measure - 2.1% versus 2.5% stillbirth rates for those with and without HbA1c (p=0.6). Higher proportions of women with T2DM achieved the target pre-pregnancy HbA1c of <53 mmol/mol compared with those with T1DM in both the live and stillborn groups. However, similar to the pattern seen in the T1DM population, those who experienced a stillbirth had lower rates of achieving this target compared with the livebirth group, albeit, not statistically significant (table 4.3).

Figure 4.3 shows the mean HbA1c across each stage of pregnancy for those who had live and stillborn infants. There was no association between gestational glycaemia and stillbirth. In contrast, preconception HbA1c was 12 mmol/mol higher in the stillbirth group (p = 0.01).

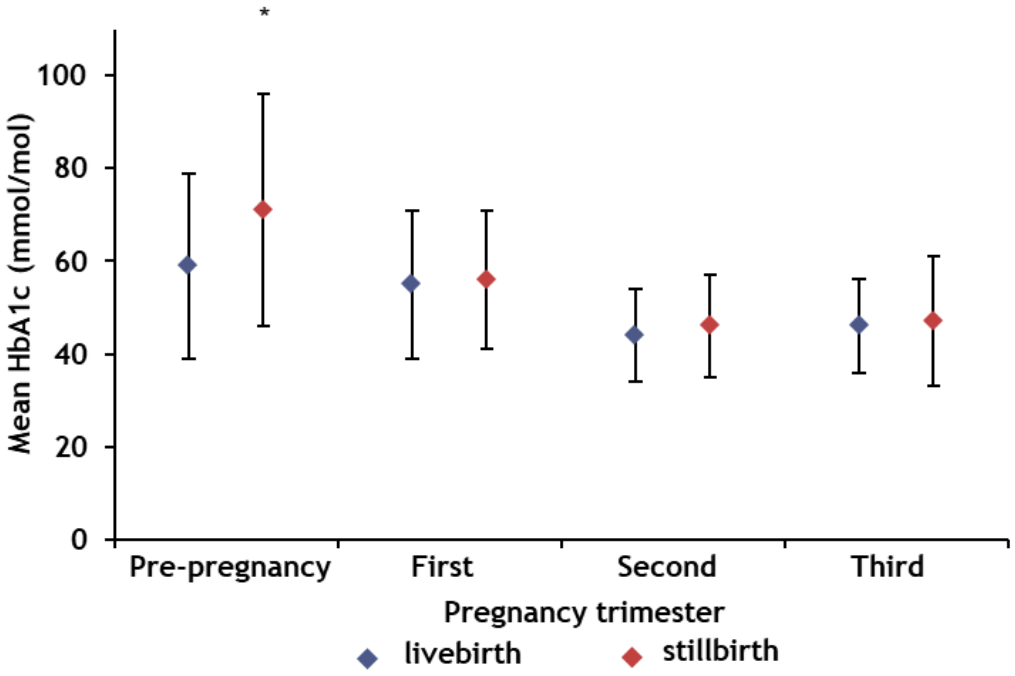


Figure 4.3: Mean HbA1c in mothers with T2DM at each stage of pregnancy according to infant vital status at birth

Mean maternal HbA1c at each stage of pregnancy is demonstrated for the mothers who delivered a liveborn infant (blue diamond) and the mothers who delivered a stillborn infant (red diamond). Error bars demonstrate the corresponding SD.

*p<0.05 for stillbirth versus livebirth for that pregnancy stage by t-test

Number of available HbA1c values:

pre-pregnancy: 796 (50%) livebirths and 17 (46%) stillbirths; 1st trimester: 785 (50%) livebirths and 15 (41%) stillbirths; 2nd trimester: 775 (49%) livebirths and 16 (43%) stillbirths; 782 (50%) livebirths and 11 (30%) stillbirths.

4.3.2.2 Type 2 diabetes and timing of delivery in relation to stillbirth

In T2DM stillborn infants were delivered over 3 weeks earlier than liveborn infants (p < 0.0001). Figure 4.4 demonstrates the stillbirth rate and absolute number of livebirths according to gestational age at delivery. Similar to T1DM, stillbirths occurred across a wide range of gestations from 24 to 39 weeks. Two

thirds (68%) of stillbirths in T2DM occurred preterm (<37 weeks), and occurred at low frequency in any given gestational week. This compares to 24% of livebirths being delivered before 37 weeks. Stillbirth rates increased towards term, particularly from 36 weeks onwards and peaked in the 39th week at 9.3 (95% CI 2.4, 29.2) per 1000 ongoing pregnancies.

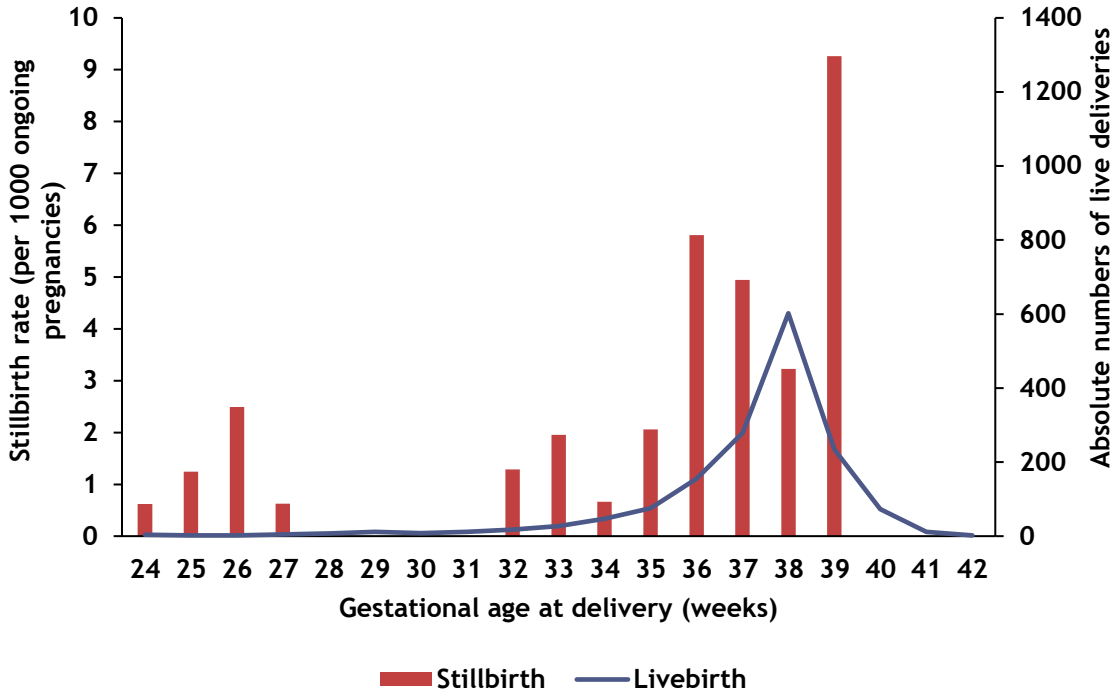


Figure 4.4: Stillbirth rate (per 1,000 ongoing pregnancies) and corresponding number of liveborn deliveries according to gestational week of delivery in T2DM

This figure shows the stillbirth rate at each gestational week expressed per 1,000 ongoing pregnancies for that particular week of gestation (x-axis) for mothers with T2DM. The red bars show the crude stillbirth rate for that gestational week with the primary y-axis (left) quantifying the rate. The blue line demonstrates the absolute number of liveborn deliveries occurring at each gestational week, for which the secondary y axis (right) is used for quantification.

4.3.2.3 Infant birthweight and stillbirth risk in type 2 diabetes

Table 4.4 categorises infants according to birthweight centiles. The majority (60%) of infants born to mothers with T2DM had birthweights appropriate for gestational age (10-90th centile), but just over a third were born LGA.

Using the 10-90th centile group as a reference, stillbirth risk appeared highest in the small for gestational age babies (3-fold higher) but was also increased in those infants who had birthweights above the 95th centile (>2-fold increase) (p=0.04).

Table 4.4: Stillbirth prevalence according to birthweight centile category in infants born to mothers with T2DM

	Birthweight centile (total n within category)			
	<10th (n=82)	**10th-90th (n=948)**	**>90th (n=570)**	**>95th (n=417)**
% stillbirth (n)	4.9% (4)	1.7% (16)	2.8% (16)	3.6% (15)
% livebirth (n)	95.1% (78)	98.3% (932)	97.2% (554)	96.4% (402)
Odds ratio (95% CI)	3.0 (1.0-9.2)	REF	1.7 (0.8-3.4)	2.2 (1.1-4.4)

Missing birthweight values: n=16

4.3.3 The relationship of glycemia and maternal BMI to birthweight and stillbirth

Given the association with birthweight and stillbirth, data relating to glycemia and its effects on birthweight in T1DM and T2DM where analysed. Higher birthweights were associated with higher HbA1c in both populations. Table 4.5 reveals the mean birthweight Z-score for infants according to the combined pre-pregnancy and third trimester HbA1c. In T1DM, mean birthweight Z-scores were 0.9 (0.12) SD higher than the reference population in the subgroup of mothers with HbA1c in the lowest quartiles for prepregnancy and third trimester HbA1c (<52 mmol/mol and <42 mmol/mol respectively). This increased to a mean birthweight Z-score of 1.73 (0.09) for those who had HbA1c values in the highest quartiles (>76 mmol/mol pre-pregnancy and >56 mmol/mol in the third trimester).

In T2DM, a similar pattern was observed. Mothers who had HbA1c values in the lowest pre-pregnancy and third trimester quartiles delivered infants 0.39 (0.13) SD heavier than the background population, but for those in the highest quartiles, this increased to 1.79 (0.26 SD).

Table 4.5: Mean birthweight Z-score according to combined pre-pregnancy and third trimester HbA1c values in T1DM and T2DM

		Type 1 diabetes				Type 2 diabetes			
		3rd trimester HbA1c quartile				3rd trimester HbA1c quartile			
		Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
Pre-pregnancy HbA1c quartile	Q1	0.90 (0.12)	1.43 (0.14)	1.62 (0.19)	1.62 (0.3)	0.39 (0.17)	0.95 (0.17)	1.55 (0.29)	2.35 (0.43)
	Q2	0.73 (0.17)	1.32 (0.11)	1.54 (0.11)	1.70 (0.15)	0.38 (0.28)	0.74 (0.23)	1.51 (0.27)	1.93 (0.39)
	Q3	0.96 (0.23)	1.29 (0.14)	1.61 (0.11)	1.80 (0.12)	0.69 (0.34)	0.97 (0.32)	1.84 (0.39)	1.52 (0.36)
	Q4	0.64 (0.27)	1.12 (0.19)	1.40 (0.13)	1.73 (0.09)	0.88 (0.36)	0.89 (0.31)	1.11 (0.29)	1.79 (0.26)

Quartiles defined by HbA1c values from both T1DM and T2DM combined.

Prepregnancy:

Quartile 1: <52 mmol/mol, Quartile 2: ≥52 mmol/mol and < 63 mmol/mol, Quartile 3: ≥ 63 mmol/mol and < 76 mmol/mol, Quartile 4: ≥ 76 mmol/mol

Third trimester:

Quartile 1: <42 mmol/mol, Quartile 2: ≥ 42mmol/mol and < 49 mmol/mol, Quartile 3: ≥ 49 mmol/mol and < 56 mmol/mol, Quartile 4: ≥ 56 mmol/mol

Univariate analysis suggested that higher HbA1c pre-pregnancy (OR 1.03 [95% CI 1.01, 1.04]; p = 0.0003) and in each trimester (OR 1.04 [95% CI 1.02, 1.05], 1.05 [95% CI 1.03, 1.07] and 1.06 [95% CI 1.04, 1.08] in first, second and third trimesters, respectively; all p < 0.0001) were significantly associated with stillbirth. Multivariate analysis adjusted for maternal age, SIMD score and diabetes duration, showed that these effects remained for individual timepoints. However, when pre-pregnancy and third trimester HbA1c were added to the model (n=1382 for all variables), only third trimester HbA1c remained associated with stillbirth (OR 1.05 [95% CI 1.02, 1.08]; p=0.0008). The effect from pre-pregnancy HbA1c was attenuated in this model (OR 1.02 [95% CI 0.99,1.04]: p=0.08).

In univariate models, weaker effects were also seen from shorter diabetes duration (OR 0.96 [95% CI 0.93, 0.99]; p = 0.01) and lower maternal BMI (OR 0.92 [95% CI 0.86, 0.99]; p = 0.04).

In T2DM, univariate analysis revealed associations with stillbirth and higher BMI (OR 1.07 [95% CI 1.01, 1.14]; p=0,02) and higher pre-pregnancy HbA1c (OR 1.02 [95% CI 1.00, 1.04]; p=0.016). In contrast to T1DM, HbA1c at later stages of pregnancy did not have significant associations. The effects from BMI and pre-pregnancy HbA1c remained in multivariate models adjusted for maternal age, SIMD score and diabetes duration. BMI appeared to have effects independent of the pre-pregnancy HbA1c with an OR 1.09 [95% CI 1.02-1.17], p=0.01 when both terms added to this multivariate model. Pre-pregnancy HbA1c also remained to have a significant and independent effect on stillbirth risk (OR 1.03 [95% CI 1.01, 1.05], p=0.006).

4.3.4 Regional variation in birthweight, timing of delivery and stillbirth outcomes in pregnancy complicated by diabetes

There were no significant differences in stillbirth rates by health board area of delivery (p = 0.60) or by delivering hospital unit size (p = 0.39). There were however observed regional differences in gestational age at delivery in both T1DM (range 33.4 ± 1.6 to 36.9 ± 0.2 weeks across all health boards, p < 0.0001) and T2DM (range 36.4 ± 0.3 to 37.8 ± 0.2 weeks across all health boards, p = 0.006). In case of skew, the smaller health boards delivering <5 women with diabetes per annum were then excluded as described in chapter 2.1.6 and data reanalysed. The range of gestational ages was more constricted using this method, but significant variation remained between health boards for both T1DM (range 36.1 ± 0.1 to 36.9 ± 0.2 weeks, p < 0.0001) and T2DM (range 36.5 ± 0.3 to 37.8 ± 0.2 weeks, p = 0.007). Figure 4.5 demonstrates gestational age at delivery according to anonymised health board regions. There was also an effect of hospital unit size on gestational age at delivery in T1DM, with the largest units delivering infants on average 4 days earlier than those delivering moderate

numbers of women with diabetes (10-19 per annum), p=0.02. Similar effects were not seen in T2DM (figure 4.6).

There was also significant regional variation across health boards in mean birthweight Z-scores in T2DM (range: 0.25 – 1.46, p<0.0001), as shown in figure 4.7. No statistically significant differences were seen in the T1DM population. Hospital unit size did not appear to have an effect on birthweight in T1DM or T2DM (p=0.2 for both in a model that included smoking, maternal age, year of delivery, duration of diabetes and deprivation scores).

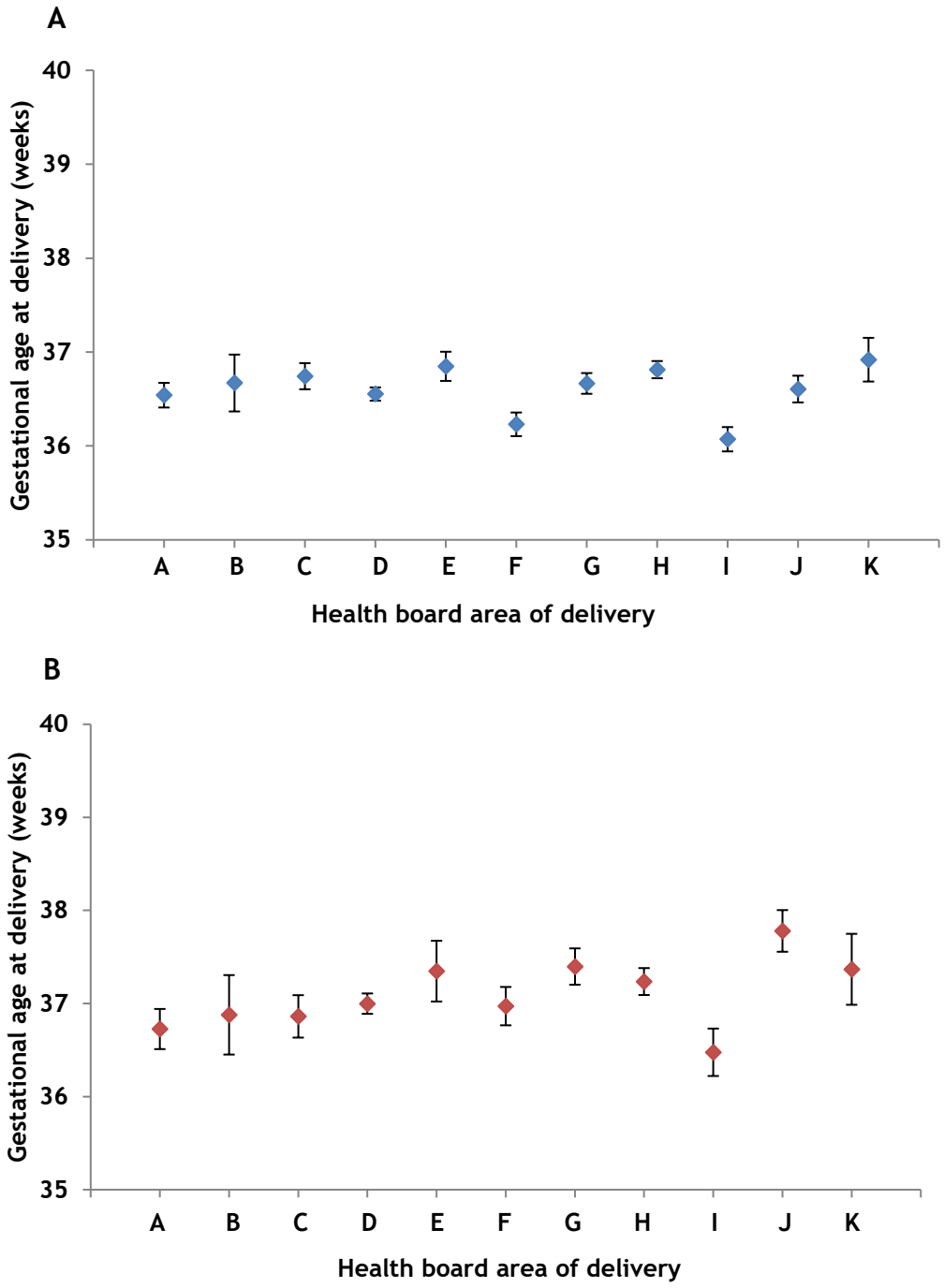


Figure 4.5: Mean gestational age at delivery of infants born to mothers with T1DM (panel A) and mothers with T2DM (panel B) according to health board area of delivery

Panel A shows the mean gestational age at delivery in weeks (y axis) of infants born to mothers with T1DM according to the health board in which they delivered in (anonymised A-K on the x axis). The same information relating to pregnancies complicated by T2DM is shown in panel B. The coloured diamond displays the mean gestational age, with corresponding error bars demonstrating the standard error of the mean. There was small but statistically significant variation across health boards in T1DM (36.1 ± 0.1 to 36.9 ± 0.2 weeks, $p < 0.0001$), with wider variation in T2DM (36.5 ± 0.3 to 37.8 ± 0.2 weeks, $p = 0.007$).

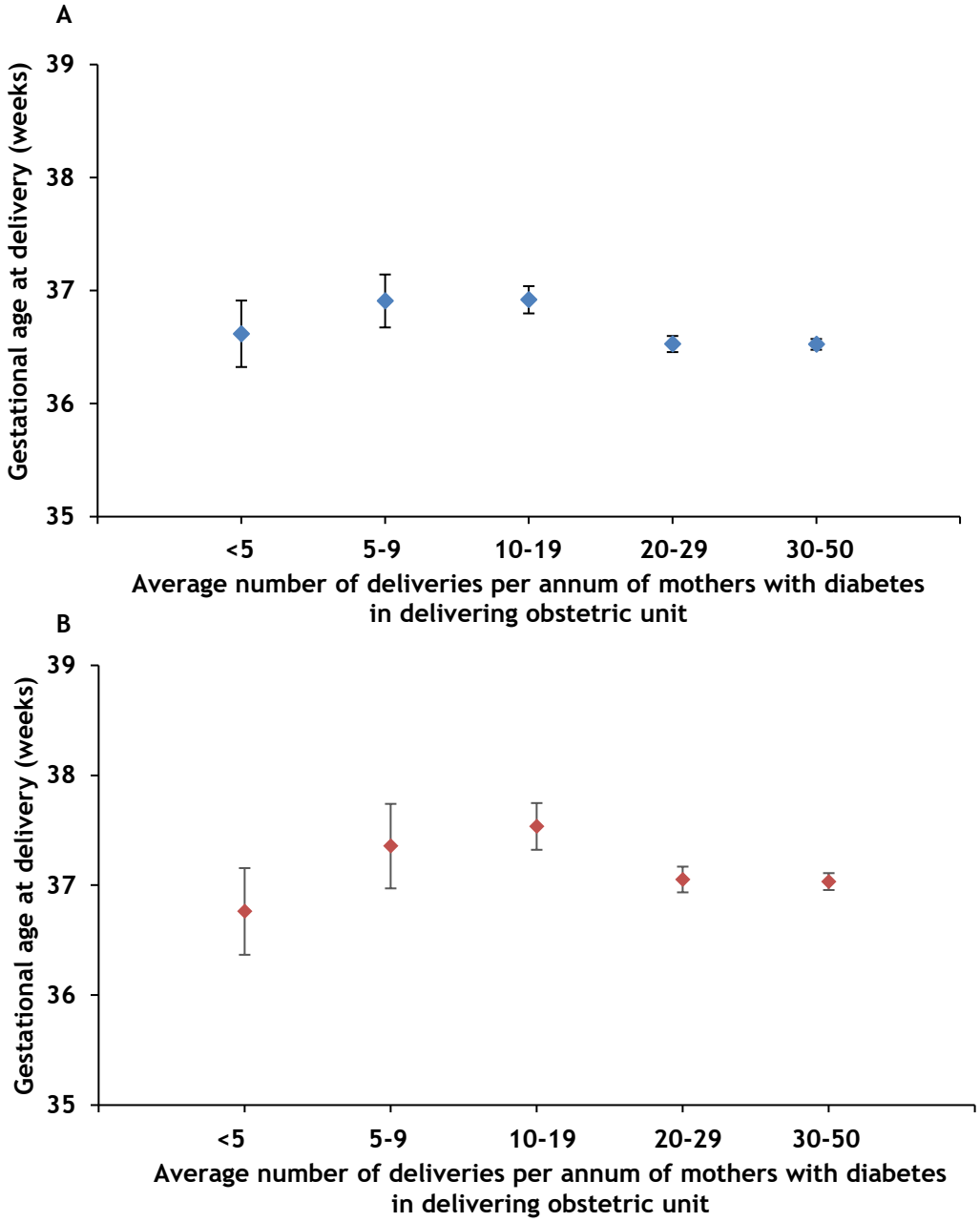


Figure 4.6: Mean gestational age at delivery to mothers with T1DM (panel A) and T2DM (panel B) according to the delivering obstetric unit size (expressed as the number of deliveries to mothers with diabetes per annum)

Panel A shows the mean gestational age at delivery in weeks (y axis) of infants born to mothers with T1DM according to the size of the delivering obstetric unit. The size of the delivering obstetric unit is expressed in categories according to the average number of deliveries per year that occur in women with diabetes at that particular unit (shown on the x axis). Panel B includes the same information for women with T2DM. The coloured diamond (blue for T1DM, red for T2DM) displays the mean gestational age, with corresponding error bars demonstrating the standard error of the mean. In T1DM, women were delivered on average 4 days earlier if delivered in the largest unit size (30-50 deliveries per annum) versus those units delivering 10-19 diabetic pregnancies per annum (p=0.02). There was no statistical difference in average gestational age of delivery between units of varying size in T2DM deliveries (p=0.2).

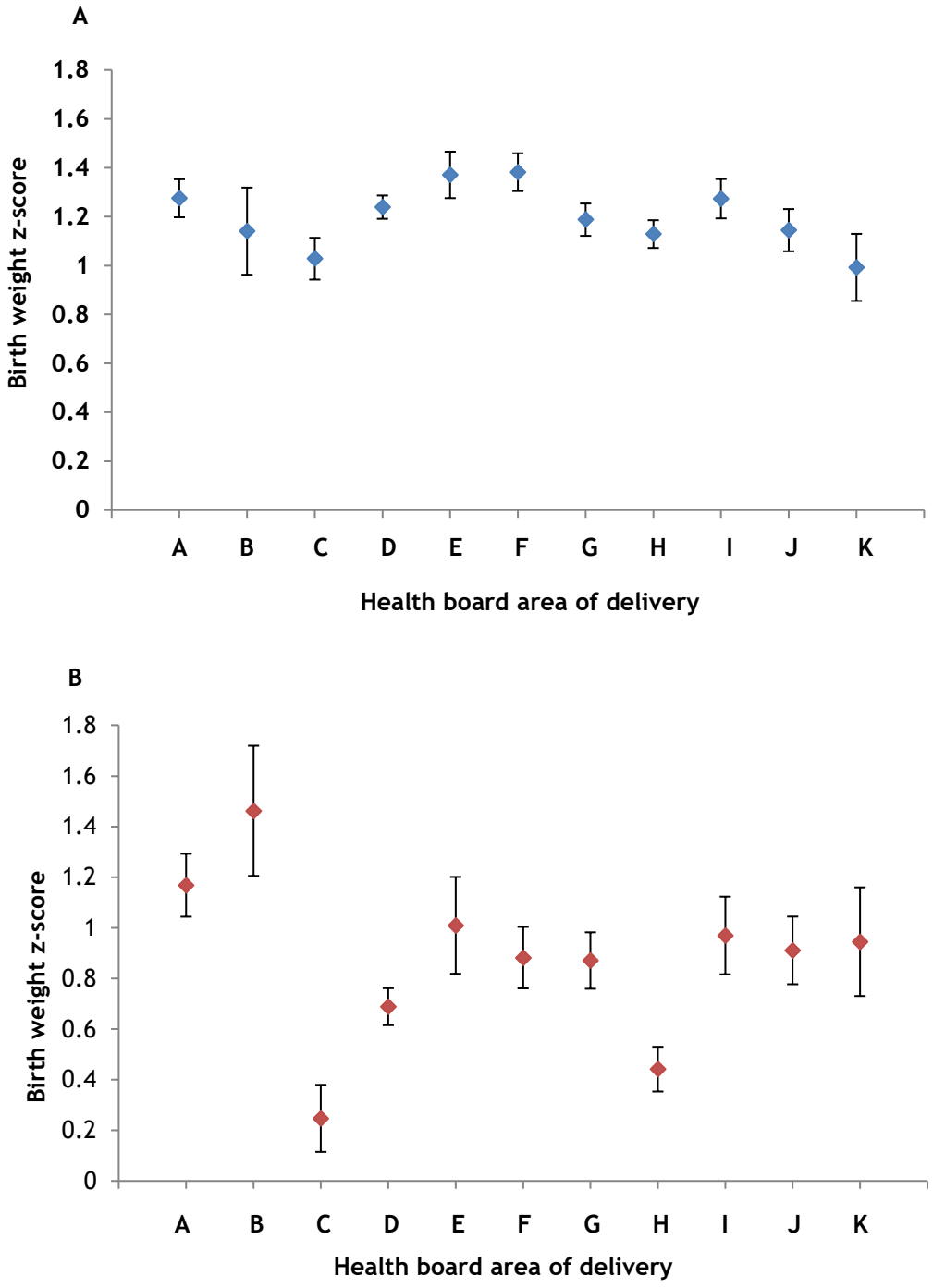


Figure 4.7: Mean birthweight Z-score of infants born to mothers with T1DM (panel A) and mothers with T2DM (panel B) according to health board area of delivery

Panel A shows the mean birthweight Z-scores (y axis) of infants born to mothers with T1DM according to the health board in which they delivered in (anonymised A-K on the x axis). The average birthweight was consistently higher than the reference population irrespective of health board in which delivery occurred, and statistically showed no difference. Panel B shows the same mean birthweight Z-scores but for mothers with T2DM. There was more regional variability in birthweight detected in the population with T2DM ($p < 0.0001$), which may reflect different unmeasured comorbidity in regions, or differences in antenatal care and outcome measures in T2DM.

4.4 Discussion

In this study, I have explored risk factors for stillbirth in a large population of mothers with diabetes. The main finding is that maternal blood glucose level is the key modifiable risk factor for adverse perinatal outcomes, including stillbirth. This is similar to findings from other smaller studies (Temple et al., 2006, Tennant et al., 2014, Pedersen and Brandstrup, 1956) and very recent data from the England and Wales population that showed HbA1c≥ 6.5% in later pregnancy as a perinatal mortality risk factor in T1DM and T2DM (Murphy et al., 2021). In T1DM, mothers who had a stillbirth demonstrated higher mean HbA1c levels across all stages of pregnancy compared with those who had livebirths, despite clear improvements in glycaemia across the course of pregnancy. However, there is considerable overlap in HbA1c values between live and stillbirth groups, making it difficult to define a level of glycaemia at which stillbirth risk critically increases. The pattern of glycaemia in women with T2DM in relation to stillbirth appears different. Interestingly, pre-pregnancy HbA1c appeared a more important predictor here, and more unexpectedly with no independent association of HbA1c levels in later pregnancy. The number of stillbirths in this group is small, however and so these findings should be interpreted with caution. However, it highlights the importance of pre-pregnancy counselling and metabolic optimisation for women with diabetes. This may be a particular area for clinical teams to focus on in T2DM, especially since uptake of preconceptual counselling is generally reported as being lower than in T1DM (Macintosh et al., 2006). Taken together, strategies to improving blood glucose levels before and during pregnancy are clearly pivotal to improving outcomes.

It was interesting that despite apparent better glycaemia, mothers with T2DM had higher stillbirth rates than those seen in the T1DM population. Similar increased risk in T2DM has been reported in other populations (Roland et al., 2005, Clausen et al., 2005). In this study, higher maternal BMI had a significant adverse association with stillbirth in T2DM. It is known from previous general

obstetric population studies that maternal obesity is an independent risk factor for stillbirth, in addition to other adversarial effects in pregnancy including preeclampsia, congenital anomalies and fetal overgrowth (Aune et al., 2014, Ray et al., 2001). Excessive gestational weight gain has also been linked with increased risk (Yang et al., 2017, Yao et al., 2017, Institute of Medicine, 2009). I would have been interested to explore this further, but SMR02 lacks standardised data relating to pregnancy weight gain. Other social factors have also been implicated in stillbirth risk from studies conducted in the general obstetric population. These have demonstrated advancing maternal age (Flenady et al., 2011), nulliparity, maternal smoking and social deprivation to confer additional risk (Flenady et al., 2011, Gardosi et al., 2013). Data from 2014-2018 in England and Wales population showed higher deprivation to be associated with perinatal mortality (Murphy et al., 2021). These social factors were not formally statistically significant in their effects on stillbirth in this study, but it would be wrong to conclude absolutely no effect on risk. Overall, they are likely to contribute to wider maternal health and potentially impact access to or use of obstetric services, and therefore remain important to consider when developing strategies to improve outcomes.

One of the limitations of this study, is that the exact causes of stillbirth in this population are not known. In diabetes, at least some cases will reflect underlying congenital anomaly, the risk of which is associated with HbA1c early in pregnancy. Current Scottish guidelines recommend women with diabetes achieve an HbA1c <53 mmol/mol three months before pregnancy, in order to reduce the anomaly risk (Management of Diabetes (Scottish Intercollegiate Guidelines Network), 2017). Overall rates of women achieving target were disappointingly low in the Scottish cohort, and even lower for the stillbirth groups. However, in keeping with other studies, we would expect that most stillbirths were non-anomalous, and instead caused by metabolic effects on fetal growth and placental function (Rackham et al., 2009, Edwards et al., 2013). Future work linking the attributed cause of death from post-mortem and placental pathology with maternal antepartum metabolic markers is planned to explore aetiology further.

With this in mind, I was interested to know if fetal characteristics could help us predict those babies at risk of stillbirth. General obstetric studies have shown that babies at extremes of growth centiles are at increased risk of stillbirth (Contag et al., 2016). IUGR specifically has been identified as one of the strongest indicators of stillbirth risk, with risk increased 4.0-fold when IUGR detected antenatally, and 8.0-fold if undetected pre-delivery (Gardosi et al., 2013). My data shows a similar risk profile in the population with diabetes with absolute risk of stillbirth highest in SGA infants. This is most striking in the T1DM mothers, albeit numbers of SGA infants in this study were small (<2% of T1DM births, 5% T2DM). Babies born very LGA with birthweights above >95th centile were also at increased risk in T2DM, which reflects similarly to general obstetric population data. (Contag et al., 2016). This was less apparent in the T1DM group where a higher proportion (40%) of infants were born in this weight category. Routine obstetric care for women with diabetes recommends regular growth scans from 28 weeks of gestation to help identify at-risk pregnancies and allow decision-making around earlier delivery where appropriate (Management of Diabetes (Scottish Intercollegiate Guidelines Network), 2017). Given this likely pattern of obstetric intervention in the Scottish population, similar rates of stillbirth seen in higher birthweight infants may represent some success of these policies. It is also noticeable that stillborn babies had similar adjusted birthweights to liveborn infants (both T1DM and T2DM). In the datasets used, birthweight was the only available standardised measure of growth. Specifically, data from antenatal growth assessment were lacking and as a result, using birthweight as an indicator of fetal growth, may have significantly underestimated growth in the stillbirth group in which it may have stopped or faltered pre-delivery. It is widely accepted that fetal overgrowth relates to maternal hyperglycaemia in later pregnancy. However, it is interesting that even in the subgroup of women with HbA1c in the lowest quartiles pre-pregnancy (<52 mmol/mol) and in the third trimester (<42 mmol/mol), birthweight remained considerably higher in pregnancy complicated by diabetes compared to the general obstetric population.

Optimal timing of delivery in pregestational diabetes is debated and practice varies internationally. In Scotland, routine delivery is recommended between 38 and 40 weeks, while the American College of Obstetricians and Gynaecologists suggests delivery in the 39th week (Pregestational Diabetes (American College of Obstetricians and Gynaecologists), 2018, Management of Diabetes (Scottish Intercollegiate Guidelines Network), 2017). In England and Wales, the newest NICE guidelines suggest earlier delivery during the 37th or 38th week (Diabetes in Pregnancy (National Institute for Health and Care Excellence), 2015). All guidelines recommend individualised assessment for expedited delivery in those with additional complications or obstetric risk. As mentioned previously, part of the rationale to earlier delivery involves avoidance of stillbirth in at-risk pregnancy. In two prior series from the UK, stillbirth risk in women with diabetes compared with the background population was increased across all stages of pregnancy, but significantly so to at least fivefold at term (Tennant et al., 2014, Holman et al., 2014). In my data, a third of the stillbirths occurred at term, the majority of which would be expected to be antepartum rather than intrapartum. While it is facile to observe that earlier delivery might have avoided these, decisions to expedite delivery have to be balanced against increased risk of complications such as neonatal RDS at earlier gestations (Stutchfield et al., 2005). Using data from the general obstetric populations, I could speculate that routine delivery of this study population at 37 weeks with expectant glucocorticoid administration for fetal lung maturation, could have potentially prevented 22 stillbirths in T1DM (resulting in stillbirth rate 10.3 per 1,000 births) with resultant 142 cases of RDS. In T2DM, there may have been prevention of 12 stillbirths (resulting in stillbirth rate 15.5 per 1,000 births) but with 72 cases of RDS. For some, this may seem an acceptable balance of risk, however, the risk of neonatal morbidity would need to be more formally explored before recommendations for optimal timing of delivery could made, especially considering the very low representation of mothers with diabetes in current studies (Stutchfield et al., 2005). Furthermore, RDS is up to five times more common in pregnancy complicated by diabetes and surfactant production is impaired with hyperglycaemia (Robert et al., 1976, Yildiz Atar et al., 2021). It may also be that mothers with diabetes who are able to achieve near

normoglycemia have a different balance of risk compared to those who have greater glucose excursions. Whilst further research in this area is warranted, it is also important to note that the majority of stillbirths in diabetes are occurring preterm. In these cases, routine earlier delivery would cause harm and be less likely to prevent neonatal morbidity, at least until more accurate methods of predicting risk become available.

Studies in other UK nations have suggested significant regional variation in delivery of care to women with diabetes in pregnancy (Murphy et al., 2017). In Scotland, almost all care is organised in multidisciplinary clinics based in secondary (hospital) care. While units are of differing sizes, stillbirth rates do not appear significantly different. This is reassuring, although the study is underpowered to detect this in a meaningful and robust way. There was notable variation in other important obstetric outcome measures, namely gestational age at delivery and birthweight. Some of this may reflect migration of complex cases to larger centres for delivery, but may also indicate regional variation in obstetric antenatal care and delivery policies.

Finally, it was unexpected that stillborn infants were significantly more likely to be male in the T2DM population. It has previously been acknowledged in general obstetric studies that male foetuses are more vulnerable in utero, but mortality risk is only reported as moderately increased by 10 % (Mondal et al., 2014). Yet, in this study, the crude stillbirth rates are fourfold higher in male infants than in females for T2DM (36.3 per 1000 male births vs 8.8 per 1000 female births). Male fetuses have higher metabolic demand in the later stages of pregnancy and have smaller placentas than females, which may mean less compensatory reserve (Dearden et al., 2018, Eriksson et al., 2010). The cumulative effects of a vulnerable placenta at risk of vasculopathy due to diabetes, higher metabolic demand of the male infant, coupled with hyperglycaemia-driven fetal overgrowth might explain the observed increased risk. There may also be detrimental effects on placental function from hyperglycaemia, a mechanism which will be explored later in this thesis (chapter 6).

There are major strengths from this study. Mainly, it includes whole-population data that is largely free from selection bias and has been shown in quality assurance to be robust. However, it is observational data and does have inherent shortcomings. The population being investigated will have undergone obstetric intervention, such as earlier delivery, which may impact on risk factor estimation at specific timepoints. Data on glycaemia is also only available in 50-60% of cases, and so interpretation of available data may not be applicable to the remaining 40% of the population in whom data was not measured. Also, HbA1c measures after 2006–2008, would have been from DCCT-aligned laboratories but prior to these dates may have shown regional laboratory variation. This study used HbA1c as measure of glycemia, but it is important to acknowledge that it is not routinely recommended to be measured in later pregnancy, although many clinicians do. In later pregnancy, it becomes a less robust correlate of maternal blood glucose level and can often underestimate glycaemia (Management of Diabetes (Scottish Intercollegiate Guidelines Network), 2017, Hughes et al., 2016). However, despite its limitations, HbA1c remains of some value even in later pregnancy, and the glycaemia results in this study complement others (Edwards et al., 2013, Lauenborg et al., 2003, Mathiesen et al., 2011, Mathiesen, 2016).

It would also have been interesting to include information on microvascular complications but standardised data relating to these were not readily available within the database. Nephropathy has previously been recognised to increase the risk of both IUGR and stillbirth (Lauenborg et al., 2003, Ekbom et al., 2001). Methodologies for detecting microvascular renal disease are numerous (e.g., urinary albumin creatinine ratio, urine albumin concentration etc) and changed over the audit period in our population, limiting the ability to accurately report prevalence. Aspects of prepregnancy counselling uptake such as high dose folic acid supplementation (to prevent neural tube anomalies) would also have been of interest, as would have exposure to potentially teratogenic drugs. Robust prescription information however was not available for most years of the study. Finally, preeclampsia is a risk factor for IUGR and stillbirth (Persson et al., 2009, Duley, 2009, Dunne et al., 2003). Preeclampsia is known to be underreported in

these routine data, and consistent with this it affects 6.1% of our T1DM and 4.5% of our T2DM cohorts, which is lower than other population estimates (Roberts et al., 2011)[44].

In conclusion, maternal glycaemia and BMI are the main modifiable risk factors associated with stillbirth in pregnancy complicated by diabetes. However, there is significant overlap in values between live and stillborn groups making it difficult to predict exactly which pregnancies are at risk. Achievement of near normoglycaemia is likely key to minimising risk, and methods of supporting women to achieve this in pregnancy are important. Programmes designed to support maternal weight optimisation pre-pregnancy may also help to reduce risk but can be challenging to implement successfully. In my opinion, public health programmes should focus on cardiometabolic health in women of childbearing age, as a potential strategy to improve both pregnancy outcomes and longer-term health of mothers and their offspring. Better prediction methods of at-risk pregnancies are crucial to improving outcomes, but until this becomes available, earlier delivery may be an attractive option to reduce term stillbirth and should be researched further.

5 Chapter 5: Maternal vascular function in pregnancy complicated by diabetes

5.1 Introduction

Given the notable relationship of higher maternal glycaemia with adverse pregnancy outcomes in the population with T1DM and T2DM, it seems pertinent to explore maternal and placental physiology in pregnancy complicated by diabetes. Numerous studies have explored physiological differences in adverse outcome pregnancy, but importantly, few studies involve, or are specific to mothers with diabetes.

In healthy pregnancy, various maternal vascular adaptations occur from early gestation to support the development and optimal perfusion of the uteroplacental circulation, which in turn supports growth of the developing fetus. Physiological changes in pregnancy are discussed in more detail in chapter 1.2 but in summary, these include a large expansion of maternal circulatory volume accompanied by significant systemic and even larger uterine vasodilation. This in turn increases cardiac output and maximises uteroplacental flow (Boeldt and Bird, 2017, Sanghavi and Rutherford, 2014). Maternal vasodilatation is of particular importance, and is mediated through several complex endocrine and neuronal pathways. There are ongoing controversies in exact mechanisms controlling maternal vascular tone during pregnancy, but endothelial function appears to play some role (Dørup et al., 1999). Notably, a progressive increase in endothelium-dependent flow-mediated vasodilatation is observed in healthy pregnancy compared to non-pregnant women (Dørup et al., 1999, Lopes Van Balen et al., 2017, Quinton et al., 2007). FMD brachial artery is a non-invasive measure of endothelium-dependent vasodilatation induced by intraluminal hyperaemia post-ischaemia in macrovascular beds. Coronary epicardial vasoreactivity to endothelium-dependent agonists infused during coronary angiography is the “gold standard” method to assess endothelial function but is undesirable due to its invasive nature. This is particularly true in pregnancy where non-invasive tests are preferred. FMD brachial artery

correlates well with coronary epicardial vasoreactivity and is the most widely used non-invasive test for endothelial function (Corretti et al., 2002, Flammer et al., 2012). Studies have also utilised a variety of other endothelial measures such as digital pulse wave tonometry post-ischaemia and circulating endothelial biomarkers.

Pregnancies with adverse outcomes, such as in preeclampsia and PIH have reduced endothelial function compared to healthy pregnancy (Roberts and Redman, 1993, Knock and Poston, 1996, Fitzgerald et al., 1987). The evidence base is strong for pregnancies complicated by preeclampsia, but other complications of pregnancy have also been studied. Women who develop preeclampsia have been shown to have reduced FMD brachial artery in the second and third trimesters compared with healthy pregnant controls (De Resende Guimarães et al., 2014, Savvidou et al., 2003), and importantly, have been shown to have endothelial dysfunction that predates the clinical syndrome (Savvidou et al., 2003, Weissgerber et al., 2016). Women who develop PIH have also been shown to a have a smaller rise in urinary metabolites of the endothelially derived vasodilator prostacyclin during pregnancy, suggesting a role of altered endothelial function in PIH (Fitzgerald et al., 1987). Growth restricted and placental insufficiency pregnancies have been less studied. One group reported increased expression of the endogenous NOS inhibitor, asymmetric dimethylarginine in pregnancies that showed ultrasound evidence of increased placental vascular resistance (Savvidou et al., 2003), and we know from histopathological studies that placental vascular insufficiency is extremely common in pregestational diabetes (Evers et al., 2003). Another study showed reduced maternal vasodilatory capacity (endothelium-dependent and -independent) in growth restricted pregnancies (Savvidou et al., 2003). Many studies exploring mechanisms of adverse pregnancy outcome exclude women with diabetes, due to potential confounding effects on vascular function. Yet, we know that women who have diabetes have significantly higher rates of these disorders. Preeclampsia affects approximately 10-20% of pregnancies complicated by diabetes, rising to as much as 64% in those with diabetes complications such as severe microangiopathy (Ekbom et al., 2001, Weissgerber

and Mudd, 2015). We also learned from the landmark HAPO study that there is a linear relationship of maternal glycaemia with risk of preeclampsia, even at apparently mild hyperglycaemia levels (Metzger et al., 2008). Taken together, it would seem plausible that hyperglycaemia in pregnancy may assert effects on maternal vasculature that increase the risk of cardiovascular complications in pregnancy, and has the potential to affect uteroplacental perfusion.

There is a wealth of evidence showing longstanding diabetes, and specifically toxic effects of hyperglycaemia, to be associated with endothelial dysfunction and subsequent cardiovascular disease (Avogaro et al., 2011). Research into the effects of diabetes and hyperglycaemia on endothelium during pregnancy is scarcer with conflicting results amongst the few that do exist. Ramsay et al showed that in pregnancy complicated by T1DM, microvascular vasodilatory capacity was diminished in response to endothelium-dependent (acetylcholine) and endothelium-independent (sodium nitroprusside) agonists, and importantly the difference in acetylcholine response was attenuated when adjusted for HbA1c suggesting that glucose had detrimental effects on endothelium (Ramsay et al., 2003). In contrast, another study by Ang et al showed no difference in endothelial reactivity of maternal subcutaneous adipose arteries when exposed to vasodilating agonists during wire myography experiments (Ang et al., 2002). In GDM the studies are similarly conflicting. One small study looking at temporal trends of FMD brachial artery across pregnancy complicated by GDM showed women with GDM to have similar FMD values to non-diabetic pregnant controls (Garg et al., 2017). This differs to published work by Guimaraes et al which demonstrated women with preeclampsia or GDM had significantly lower FMD% values than healthy controls, albeit this included a wide range of gestations for all groups (23-39 weeks) (De Resende Guimarães et al., 2014).

We also know that circulating placentally-derived angiogenic factors, such as sFlt1, PlGF and TNF-α are disrupted in hypertensive and growth restricted pregnancies (Zeisler et al., 2016, Saarelainen et al., 2012, Åsvold et al., 2011). Specifically, the balance between pro-angiogenic factors such as PlGF, and anti-angiogenic peptides such as sFlt-1 appear to be an important determinant of

placental vascular health, and subsequent pregnancy outcomes. Low PlGF values (<5th centile measured using Triage™ system) has high sensitivity for predicting imminent delivery due to preeclampsia in women presenting with suggestive symptoms presenting before 35 weeks gestation, and was also found to be associated with very growth restricted pregnancy (Chappell et al., 2013). A further landmark preeclampsia study showed sFlt1:PlGF ratio <38, was useful in predicting absence of the condition (Zeisler et al., 2016). Again, these studies include small numbers of women with diabetes. The UPBEAT research consortium specifically explored the explored GDM pregnancy and suggested that PlGF may not be as reliable in prediction of preeclampsia in obese GDM women versus obese non-GDM (Vieira et al., 2018). However, PlGF was measured at a much earlier time point in pregnancy. They suggest increased proinflammatory markers in diabetic pregnancy as an alternative mechanism of preeclampsia development. This work has therefore also included exploration of these angiogenic biomarkers and inflammatory cytokines, to determine if they are dysregulated in diabetic pregnancy (irrespective of preeclampsia development), and any association with altered vascular function in hyperglycaemic pregnancy.

The primary aim of this study was therefore to establish if maternal endothelial dysfunction existed in women with GDM pregnancy and if there was any relationship with maternal glycaemia and vascular function in this group. As a secondary measure, the association with endothelial function and circulating angiogenic factors (sFlt-1, PlGF) and inflammatory cytokines (IL-6, IL-1β, TNF-α) in GDM and non-GDM pregnancy were investigated.

Primary outcome:

- FMD% brachial artery in GDM versus control groups

- Serial change in FMD from baseline in treated GDM versus control groups

Secondary outcomes:

- Maternal serum angiogenic factors (sFlt1, PlGF) in GDM versus control groups

- Maternal serum inflammatory cytokine concentrations (TNFα, IL-6, IL-1ß) in GDM versus control groups

5.2 Materials and Methods

5.2.1 Participant recruitment and study visit schedule

Women were recruited as outlined in chapter 2.2.3. In summary, there were three groups of women. 30 women diagnosed with GDM according to IADPSG criteria, 15 women with risk factors for GDM but normal GTT on routine clinical testing, and 15 healthy, pregnant controls without risk factors for GDM.

Women were eligible for inclusion if they were over 18 and had a singleton pregnancy. They were excluded if they had multiple pregnancy, current obstetric or placental problems, significant past medical history or medication use that would be likely to influence vascular function tests.

All women attended four antenatal study visits as below.

- Visit 1: largely occurred 24-30 weeks gestation (mean: 196 days, range: 169-212), with one participant outlying with entry to study at 220 days gestation.

- Visit 2 between 32 and 33 weeks gestation (mean: 231 days, range 224-237 days)

- Visit 3 between 34 and 35 weeks of pregnancy (mean: 241 days, range 235-245)

- Visit 4 a further two weeks later between 36 and 37 weeks of pregnancy (mean: 256 days, range 251-264).

Women attended each study visit during which information pertaining to current and past medical history and maternal demographics was obtained.

At each visit, BP and FMD brachial artery measurements were taken on the UNEX EF 38G™ semiautomated machine. FMD protocol is outlined in chapter 2.2.5. All FMD images were manually analysed by the principal researcher Sharon Mackin using UNEX EF PC analysis software (UNEX Corporation, Nagoya, Japan), and measurements taken for rest diameter (mm), maximum diameter (mm), % FMD and time to maximum dilatation (seconds)

Fasting blood samples were obtained at the first and fourth visit. EDTA samples were collected for measurement of HbA1c and fasting plasma insulin. SST samples were collected for measurement of serum fructosamine, albumin, lipid profiles, angiogenic factors (sFlt-1, PlGF) and inflammatory cytokines (IL-6, IL-1ß and TNF-α). Full details of sample handling, storage and assays can be found in chapter 2.2.6.

5.2.1.1 Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics v 22.0. GraphPad Prism v 8.0 was used to generate standard curves using logistic regression (5-PL or 4-PL if ambiguous fit on 5-Pl curve) and corresponding analyte concentrations for the Luminex™ biochemical analysis.

Differences in maternal demographics between groups were compared using one way ANOVA for continuous variables, and χ^2 for categorical variables. Data relating to time trends of BP and FMD measurements were assessed using linear mixed models that included diabetes diagnosis and gestational age (in days) as fixed effects, with participant ID incorporated as a random effect. For biochemical analysis that was not normally distributed (assessed by Q-Q plots

and Shapiro Wilk test), median values are presented and differences between groups assessed by non-parametric tests.

A p-value of <0.05 was considered statistically significant.

5.3 Results

5.3.1 Baseline characteristics

In total, 64 women were recruited, of which 4 declined ongoing participation after their first (n=3) or second visit (n=1). 30 women with gestational diabetes, 15 women with risk factors but normoglycemia (defined by screening OGTT) and 15 healthy controls continued participation through to delivery achieving targeted recruitment numbers. Baseline characteristics of these mothers at study entry (24-30 weeks) are shown in table 5.1.

Table 5.1: Baseline characteristics of mothers at study entry (visit 1: 24-30 weeks gestation)

	Gestational Diabetes (n=30)	Risk factors (n=15)	Pregnant controls (n=15)
Mean maternal age, years (SD)	31.9 (4.9)	31.1 (4.3)	32.2 (5.8)
Mean early pregnancy BMI, kg/m^2 (SD)	31.7 (7.5)**	30.0 (6.9)	24.6 (3.3)
Ethnicity, % (n)			
White UK	50.0% (15)*	73.3% (11)	100% (15)
Other White European	6.7% (2)	13.3% (2)	0% (0)
South Asian	26.7% (8)	6.7% (1)	0% (0)
Middle Eastern	0.0% (0)	6.7% (1)	0% (0)
Black African	10.0% (3)	0.0% (0)	0% (0)
Chinese	6.7% (2)	0.0% (0)	0% (0)
Primiparous, % (n)	40.0% (12)*	60.0% (9)	73.3% (11)
Maternal Smoking, % (n)			
Never	76.7% (23)	93.3% (14)	73.3% (11)
Ex	20.0% (6)	6.7% (1)	26.7% (4)
Current	3.3% (1)	0.0% (0)	0.0% (0)
Mean BP, mmHg (SD)[a]	105/63 (16/9)ł	112/71 (17/14)	104/64 (14/10)
Aspirin use, % (n)	30.0% (9)	26.7% (4)	13.3% (2)
Low molecular weight heparin use, % (n)	3.3% (1)	0.0% (0)	6.7% 91)
Multivitamin use, % (n)	76.7% (23)ł	100.0% (15)	93.3% (14)

*p<0.05, ** p<0.01 versus control group by one way ANOVA or χ^2 as appropriate

ł p<0.05 versus risk factor group by one way ANOVA or χ^2 as appropriate.

[a] Statistical difference in DBP only

Of note, women with GDM were of higher BMI and higher parity than the control group (*p*=0.004 and 0.035 respectively) but similar to those in the risk factor group. GDM mothers were also more ethnically diverse, in particular with higher numbers of South Asian women represented compared to controls (*p*=0.02), reflecting the higher GDM risk in this ethnic group.

Aspirin use remained unchanged across the course of the pregnancy, whilst multivitamin use decreased (70.3% GDM, 93.3% risk factor, 86.7% control).

5.3.1.1 Glycaemia and diabetes treatments

The women diagnosed with GDM were diagnosed at a mean gestational age 25 weeks and 3 days (178 $\pm$ 26 days). Mean fasting plasma glucose levels at the time of screening OGTT were 5.2 (range 4.1-6.5) mmol/L with mean 2-hour post 75g OGTT levels of 7.8 (range 5.0-12.6) mmol/L.

Of the 30, 13.3% (n=4) were treated with metformin and 3.3% (n=1) with insulin at study entry. At the final study visit (week 36), this had increased to 36.7% (n=11) on metformin monotherapy, 3.3% (n=1) on insulin monotherapy and 6.7% (n=2) on combined metformin/insulin treatments.

Biochemical data for glycemia at visit 1 and 4 is shown in table 5.2. At visit 1, women with GDM had higher fasting plasma glucose (p=0.02), higher fasting insulin levels (p=0.04) and higher insulin resistance as depicted by HOMA-IR (p=0.04) compared to risk factor and control groups. Unsurprisingly, women with GDM had higher mean HbA1c (5 mmol/mol higher than controls, p<0.001, see table 5.2). At visit 4, the fasting glucose remained higher in the GDM group (p=0.01) with a trend to higher HbA1c, fasting insulin concentrations and insulin resistance scores (p=0.08 for HbA1c, p=0.1 for insulin and HOMA-IR).

Table 5.2: Maternal glycemia parameters at visit 1 and visit 4

	GDM	Risk factor	Control
Visit 1			
Number of samples	31	15	16
Fasting plasma glucose (mmol/L)	4.6 (0.4)*	4.4 (0.4)	4.3 (0.3)
Fasting plasma insulin (μU/mL)	13.7 (8.4)*	12.0 (5.2)	8.3 (3.7)
HOMA-IR	2.9 (2.0)*	2.3 (1.1)	1.6 (0.8)
Fructosamine (μmol/L)	164 (18)*	170 (16)	178 (18)
Albumin (g/L)	26 (2)	26 (1)	27 (2)
HbA1c (mmol/mol)	39 (4)***	33 (3)	34 (3)
Visit 4			
Number of samples	26	15	15
Fasting plasma glucose (mmol/L)	4.5 (0.5)**	4.1 (0.4)	4.0 (0.4)
Fasting plasma insulin (μU/mL)	14.5 (9.7)	12.0 (7.9)	8.8 (3.7)
HOMA-IR	3.0 (2.7)	2.3 (1.6)	1.6 (0.8)
Fructosamine (μmol/L)	182 (13)	186 (18)	177 (17)
Albumin (g/L)	26 (2)	26 (1)	25 (2)
HbA1c (mmol/mol)	40 (3)	37 (3)	37 (5)

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for differences between GDM and control groups by one-way ANOVA and post-hoc analysis

††† $p < 0.001$ for difference between GDM and risk factor by one way ANOVA and post-hoc analysis

5.3.2 Blood Pressure

225 of 240 (94%) planned automated BP readings were recorded in the 60 women across the study period. Reasons for missing data, were preterm delivery (n=1 at 34 weeks gestation, n=3 at 36 weeks), non-attendance of participant (n=7 at 32 week visit, 3 at 34 week visit) and 1 case of BP not recorded by researcher (34 week).

Over the course of pregnancy, 3 (5%) participants were diagnosed with PIH, all of which were in the risk factor group. Of these, 2 were treated with Labetalol. Another 3 (5%), developed preeclampsia (n=1 control at 37 weeks, 2 GDM participants at 34 and 36 weeks gestation), of which 2 were treated with Labetalol and Nifedipine at the time of study. All antihypertensive treatments were initiated by independent clinical teams, and started after the final study visit for each participant (one participant delivered preterm resulting in final

visit at 32 weeks). Mean BP at study entry was higher in women who developed a hypertensive disorder of pregnancy compared to those who did not (126/83 versus 104/63 mmHg, p<0.001). This difference was driven largely by those in the preeclampsia group - mean visit 1 BP 139/93 mmHg in the preeclampsia group versus 113/69 mmHg in those who developed PIH (p=0.03 and 0.003 for SBP and DBP respectively).

In a linear mixed model, with the mother ID incorporated as a random effect, BP did not change significantly with advancing gestational age (p=0.5 for SBP with a marginal effect in DBP (effect size 0.04 mmHg, p=0.05)). BP trends across pregnancy were no different between groups (see figure 5.1), and the small effect of gestational age on DBP was attenuated when diabetes diagnosis was included (p=0.09). There was no effect from fasting glucose or HbA1c when added to the model of gestational age and mother ID (p=0.5 for both).

5.3.2.1 Subgroup BP analysis of GDM participants

The FBG range at initial OGTT was narrow within the GDM group (4.1-6.5 mmol/L). In a mixed model that included gestational age and FBG as fixed effects, and participant ID as a random effect, higher FBG was associated with a significant increase in SBP. Every 1 mmol/L increase in FBG was associated with a 14 mmHg increase in SBP (p=0.001). This significance remained when analysis was extended to include the fasting glucose measurements taken at visit 1 and visit 4. The effect was attenuated by addition of BMI but remained significant with 9 mmHg increase per 1 mmol/L increase in FBG (p=0.03).

In a similar mixed model that incorporated the fasting glucose measurements taken at visit 1 and visit 4, gestational age and mother ID, each 1 mmol/L increase in glucose was associated with an 8 mmHg increase in DBP (p=0.005). There was also no effect of the 2-hour GTT result (range 5.0-12.6 mmol/L) on SBP or DBP, nor an effect from HbA1c.

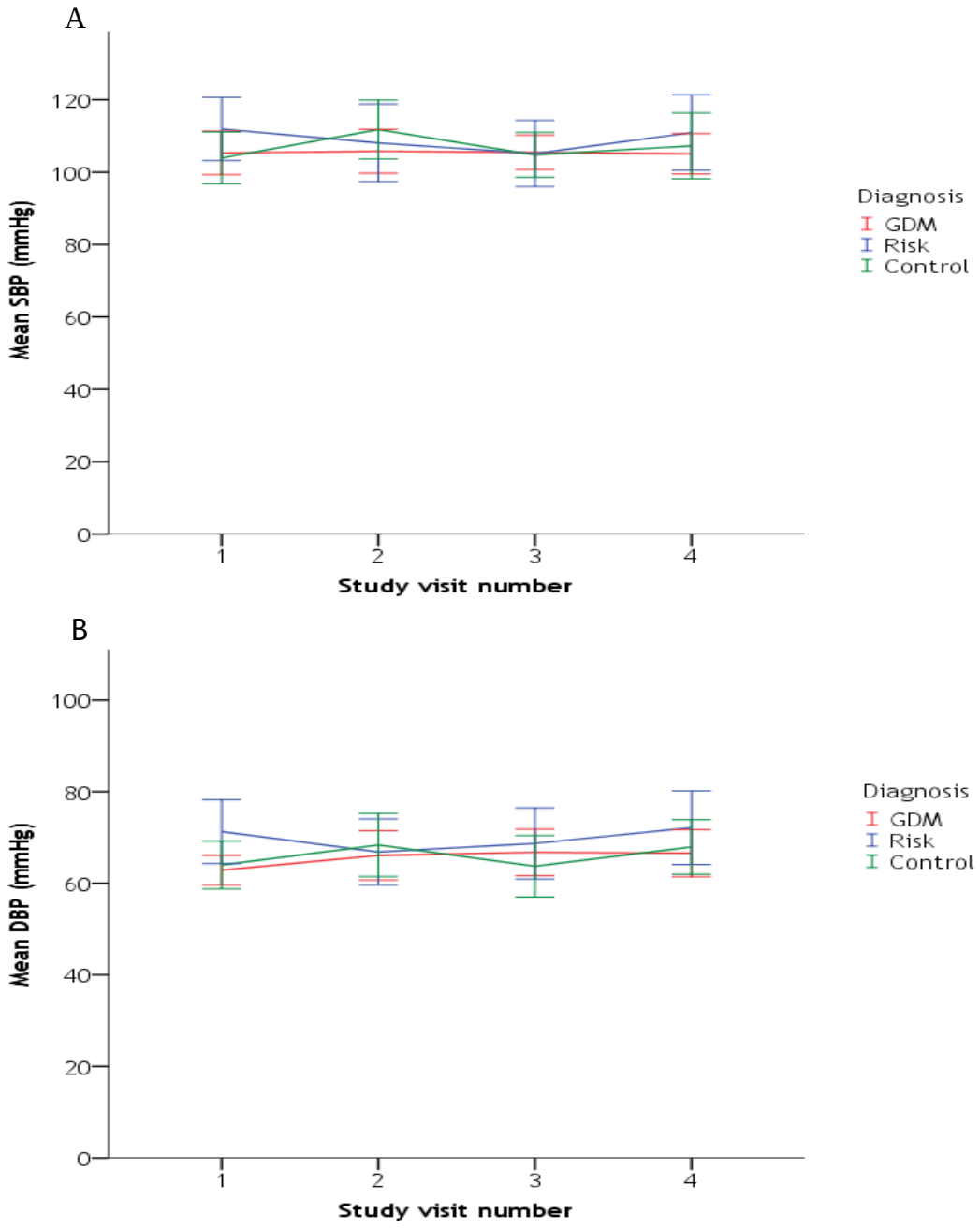


Figure 5.1: Mean SBP and DBP according to diabetes diagnosis across study visits 1-4

Figure A shows mean SBP at each study visit (visit 1: 24-28 weeks gestation, visit 2 32 weeks gestation, visit 3 34 weeks gestation and visit 4 36 weeks gestation) according to diabetes diagnosis group (GDM: red, risk factor: blue, control: green). Error bars demonstrate the SEM. Figure B shows mean ± SEM DBP for the groups. There was no change in SBP or DBP with advancing gestational age, and this pattern was no different between diagnostic categories.

5.3.3 Flow mediated dilatation (FMD) brachial artery

206 (85.8%) FMD measurements of a possible 240 were available for analysis from the 60 participants. Reasons for missing data included: 5.8% (n=10 missed visits and n=3 patients with preterm delivery completing study early as detailed in section 1.3.2) and 8.3% (n=20) FMD scans deemed of insufficient quality for analysis. This resulted in 58 of 60 participants having an adequate number of results ($\geq$3 readings across the study period or 2 readings including initial study visit and one of visit 3 or 4) to be included in further analysis; 94.8% of which had data for 3 (39.7%, n=23) or 4 visits (55.2%, n=32).

5.3.3.1 Baseline diameter and FMD % of brachial artery

The mean baseline intraluminal diameter of the brachial artery at each time point in pregnancy is shown in table 5.3 There was no significant difference in diameter between groups at any of the time points. In a model that included participant ID as a random effect, intraluminal brachial artery diameter was shown to increase with advancing gestational age (0.003mm for each day increase in gestational age, $p<0.001$). There was no difference in diameter trend between groups ($p=0.6$).

Table 5.3: Mean baseline intraluminal diameter (mm) of brachial artery according to stage of pregnancy and diabetes diagnosis

	GDM (n=27)	Risk factor (n=15)	Control (n=15)
Visit 1	3.51 (0.37)	3.39 (0.38)	3.43 (0.29)
Visit 2	3.62 (0.56)	3.38 (0.32)	3.52 (0.36)
Visit 3	3.63 (0.56)	3.54 (0.42)	3.67 (0.41)
Visit 4	3.57 (0.38)	3.56 (0.37)	3.60 (0.37)

Data shown are mean (SD)

Available data:

Visit 1: GDM n=27, Risk factor n= 15, Control n=15; Visit 2: GDM n= 25, Risk factor n=11, Control n=12; Visit 3: GDM n=23. Risk factor n=13, Control n=14; Visit 4: GDM n=23, Risk n=13, Control n=15.

FMD% did not change with advancing gestational age and was similar between groups, p=0.12 (figure 5.2), and remained non-significant after adjustment for baseline vessel diameter. There was no effect on FMD% from fasting plasma glucose (mixed model with mother ID (random effect) with gestational age and fasting plasma glucose measurements from visits 1 and 4 (fixed effects), p=0.3. There was no significant effect of fructosamine (p=0.8), HbA1c (p=0.7) nor HOMA-IR (p=0.3) on FMD% in similar models.

Similarly, there were no differences between groups in time to reach maximum vasodilatation (mean from visit 1-4 measurements: 54.3$\pm$2.2s GDM, 47.5$\pm$2.8s risk factor, 50.6$\pm$2.9s controls, p=0.1). There was no significant effect on time to maximum dilatation from fasting plasma glucose measurements (from visits 1 and 4) (p=0.3 in model including mother ID as random effect, gestational age and glucose as fixed effects). Similarly, no significant effects were seen when HOMA-IR, insulin concentrations or fructosamine were assessed in similar models.

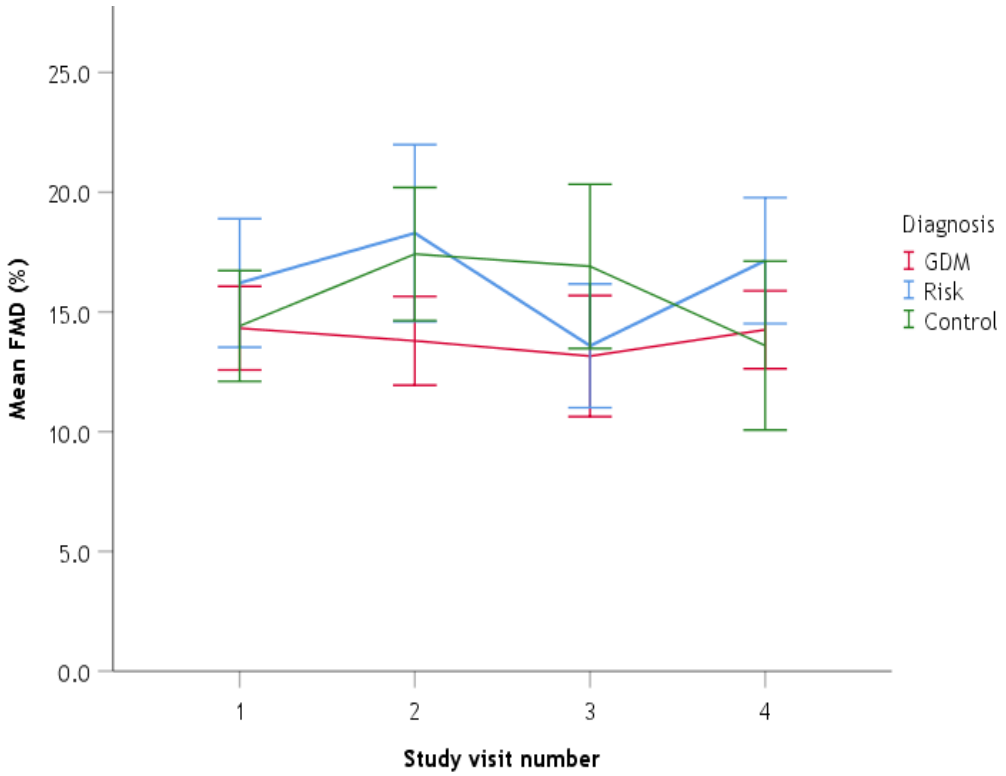


Figure 5.2: Mean flow mediated dilatation (FMD) % of brachial artery according to diabetes diagnosis across study visits 1-4

The mean flow mediated dilatation (%) of brachial artery for the GDM group (red), risk factor group (blue) and control group (green) are shown, with error bars reflecting the standard error of the mean. There is no difference evident between the groups across progressive study visits.

5.3.4 Circulating angiogenic markers and lipid profiles

5.3.4.1 Soluble fms-like tyrosine kinase 1 (sFlt-1) , placental growth factor (PlGF) and sFlt-1:PlGF ratio

The median maternal serum concentrations of sFlt-1, PlGF and the sFlt-1:PlGF ratio for each group are shown in table 5.4 The range was wide for each parameter across all groups. The sFlt-1:PlGF ratio was significantly lower in the GDM group at visit 1 (p=0.003 by independent sample median test). The median sFlt-1: PlGF ratio was numerically higher in the GDM and risk factor groups at visit 4 (p=0.1). There was no significant effect of these biochemical measures on any of SBP, DBP or FMD when tested in individual mixed models that included maternal ID, gestational age, the tested biomarker and its interaction with gestational age.

There was no correlation with PlGF and corresponding fasting plasma glucose (Pearson's r=0.05, p=0.6), fasting insulin (Pearson's r=-0.2, p=0.9) and HOMA-IR (Pearson's r=-0.1, p=0.2). sFlt-1 correlated weakly with fasting insulin concentrations and HOMA-IR measures (Pearson's r=0.211 p=0.03, and r=0.226 p=0.02 respectively). There was no significant correlation of sFlt-1 and glucose concentrations.

Table 5.4: Median concentrations and interquartile range of soluble fms-like tyrosine kinase 1 (sFlt-1), placental growth factor (PlGF) and sFlt-1: PlGF ratio in maternal serum according to diabetes diagnosis

	GDM	Risk factor	Control
Visit 1			
Median sFlt-1 (IQR) (pg/mL)	2040.5 (1472.0-2636.3)	2366.9 (1738.2-3052.6)	2435.5 (2079.4-2791.6)
Median PlGF (IQR) (pg/mL)	153.6 (74.6-358.1)	112.7 (23.6-183.1)	79.8 (33.3-141.3)
Median sFlt-1: PlGF ratio (IQR)	7.1** (4.1-14.3)	23.7 (8.5-124.1)	28.2 (18.1-141.0)
Visit 4			
Median sFlt-1 (IQR) (pg/mL)	3763.2 (2743.7-4611.8)	3018.3 (2263.9-3454.9)	3448.9 (2805.4-5308.8)
Median PlGF (IQR) (pg/mL)	67.5 (12.3-201.4)	32.2 (13.2-161.3)	110.0 (65.5-260.4)
Median sFlt-1: PlGF (IQR)	85.7 (17.9-273.1)	87.4 (15.9-171.1)	30.1 (10.2-48.6)

** $p<0.01$ via independent-samples median test

Number of available samples included based on good reproducibility of results (<20% CV on duplicate samples):

Visit 1: sFlt-1 – GDM 27, Risk factor 15, Control 16; PlGF: GDM 25, Risk factor 15, Control 14; both sFlt-1 and PlGF for ratio: GDM 21, Risk factor 15, Control 14.

Visit 4: sFlt-1 – GDM 24, Risk factor 13, Control 13; PlGF: GDM 20, Risk factor 12, Control 9; both sFlt-1 and PlGF for ratio – GDM 18, Risk factor 10, Control 7.

5.3.4.2 Tumour necrosis factor alpha (TNF-α), interleukin-6 and interleukin-1ß

The Luminex™ analysis for these three biomarkers were performed on the same samples on the same assay plates. Review of this data suggested systematic errors in the assay and so was not interpreted further. Standard curves and control samples from all three plates run showed a consistent inability to detect standard concentrations at and below 80 pg/mL (high CV% and > 30% outwith the predicted range for controls). There was a high proportion of undetectable levels of IL-6 (76% samples). In the case of IL-1ß, each plate had remarkably consistent values for samples measured on that plate suggesting measurement error. For example, plate 1 samples read within range 5.9-7.0pg/mL, plate 2 had a range from 1.9-3.5pg/mL and plate 3 had largely undetectable values.

5.3.4.3 Lipid profiles

Table 5.5 shows the mean cholesterol, HDL-cholesterol and fasting triglyceride levels for each group at visit 1 and visit 4. At visit 1, serum total cholesterol and HDL cholesterol concentrations were different between groups, with GDM having significantly lower levels of both (($p<0.01$ and <0.001 for total cholesterol and HDL-cholesterol respectively). At visit 4, total cholesterol remained lower in the GDM group ($p=0.046$ one way ANOVA, $p=0.07$ GDM versus control) with a trend to lower HDL cholesterol ($p=0.1$ GDM versus control).

There was no statistical effect seen from total cholesterol, HDL cholesterol or fasting triglycerides on BP or FMD (mixed model with maternal ID, gestational age and biochemical parameter of interest).

Table 5.5: Circulating serum lipid concentrations according to diabetes diagnosis

	GDM	Risk factor	Control
Visit 1			
Mean total cholesterol, mmol/L (SD)	4.80 (1.04)** ††	5.30 (0.60)	5.85 (1.05)
Mean HDL cholesterol, mmol/L (SD)	1.33 (0.36)*** †††	1.56 (0.36)	1.78 (0.33)
Mean fasting triglycerides,mmol/L (SD)	1.81 (0.44)	1.63 (0.47)	1.64 (0.47)
Visit 4			
Mean total cholesterol, mmol/L (SD)	5.92 (1.27)*	6.71 (1.15)	6.88 (1.42)
Mean HDL cholesterol, mmol/L (SD)	1.55 (0.44)	1.75 (0.35)	1.84 (0.50)
Mean fasting triglycerides, mmol/L (SD)	2.76 (0.90)	2.33 (0.69)	**5.57 (0.64)**

*p<0.05, ** p<0.01, *** p<0.001 between groups by one way ANOVA

†† p<0.01, ††† p<0.001 GDM versus control post-hoc t-test

Available sample numbers: Visit 1: GDM 31, Risk factor 15, Control 16; Visit 4 GDM 26, Risk factor 15, Control 15.

5.4 Discussion

5.4.1 FMD Brachial artery

Macrovascular endothelial function, as measured by FMD brachial artery in the third trimester of pregnancy is no different in GDM mothers compared with non-GDM mothers. Furthermore, there is no relationship of FMD brachial artery measures and biochemical markers of glucose control (fasting plasma glucose, HbA1c or fructosamine). However, in the GDM subgroup analysis, higher fasting glycaemia has a strong association with both SBP and DBP, suggesting that uncontrolled hyperglycaemia still exerts effects on vascular function. This

observation is in keeping with the large, multicentre studies that have shown small rises in glycemia to be associated with significant risks of developing hypertensive disorder in pregnancy (Metzger et al., 2008, Landon et al., 2009). The pathophysiological mechanism for disease remains unclear.

The FMD results are similar to a recent study by Garg et al, which explored trends in FMD across pregnancy, and whilst they found an attenuated decrease in FMD between first and third trimester in GDM versus non-GDM mothers, there was no difference in third trimester FMD values between groups (Garg et al., 2017). This contrasts with a slightly older study by Guimares which showed GDM mothers to have lower brachial artery FMD in the third trimester than non-GDM mothers (De Resende Guimarães et al., 2014). However, this study included only a single time point measurement of FMD in the third trimester, and included a wide range of gestations from 25-38 weeks so may not be directly comparable. The latter Garg study had a more comparable study design that restricted third trimester measurement to a more constricted gestational period. Furthermore, the Garg study included women with a similar severity of GDM as in my study (mean fasting levels 5.3 mmol/L and 2-hour post-75g GTT of 6.3 mmol/L), whilst the range of glycaemia included in the Guimares study was less clear. My study findings show no relationship of FMD to glycaemia, but this may not be true if women with a more severe hyperglycaemic phenotype were examined. To my knowledge, my study is the first to explore serial FMD measurements across the third trimester, and specifically the time period post-GDM diagnosis where glucose excursions would be expected to be greatest.

Whilst there was no difference in brachial artery FMD, nor the time to reach peak dilatation between groups, it would be premature to translate this as meaning no effects of glycaemia on maternal endothelial function. Using different methodologies, others have demonstrated maternal microvascular endothelial dysfunction in pregnancy complicated by diabetes (Knock and Poston, 1996, Ramsay et al., 2003), and importantly, the presence of normal microvascular function when diabetes is well controlled (Ang et al., 2002). There is a wealth of evidence that glycaemia impairs endothelial function outwith

pregnancy (Avogaro et al., 2011). Based on OGTT results, the women in this study entered with GDM that was largely on the milder scale of hyperglycaemia, albeit 40% progressed to requiring pharmacotherapy suggesting higher excursions as pregnancy progressed. Pharmacotherapy was initiated by the clinical team, and decisions made independent of study involvement. Treatments included insulin and metformin therapy, both of which could have had effects on endothelial function and confounded results. Metformin treatment has been shown to have positive effects on endothelial function through various pathways which act to increase NO availability and reduce inflammatory leucocyte adhesion (Nafisa et al., 2018). Euglycaemic clamp studies in non-pregnant volunteers have also shown that short term exposure to modest hyperinsulinemia has detrimental effects on large vessel endothelial function (Arcaro et al., 2002, Campia et al., 2004). This is likely as result of increased oxidative stress.

We know from landmark studies that microvascular beds are particularly susceptible to adverse clinical sequelae of hyperglycaemia (UKPDS 33, 1998). Perhaps due to their larger intraluminal diameter, macrovasculature requires a bigger insult before clinical or physiological effects from endothelial dysfunction become apparent. FMD has clear advantages as a test of being non-invasive and safe for mother and baby, and is accepted as standard test of endothelial function (Corretti et al., 2002). However, it has not been validated in pregnancy. There are also known susceptibilities to inter- and intra-user variability. The initial study design had hoped to improve upon this variability by using novel semi-automated technology, but myself and colleagues have since shown in our separate non-pregnancy study (Dobbie et al., 2020), that manual assessment of images provides better reproducibility. Furthermore, in this current study all primary image ascertainment and secondary analysis was performed only by me, and after several months of training so variability is likely to be minimised. Alternatively, FMD brachial artery may not be the optimal test of endothelial function in the vasodilated late stages of pregnancy. It is notable that our data suggests that women may be nearing peak vasodilatory state (in brachial artery) in these later stages of pregnancy. Additional measures of endothelial function would have been of interest. For example, peripheral

arterial tonometry (EndoPAT™) to assess microvascular beds and quantifying circulating biomarkers of oxidative stress.

5.4.2 Angiogenic and inflammatory biomarkers

The sFlt-1:PlGF ratio was lower in GDM at 24-28 week visit, with no difference seen between groups at 36 weeks. The difference in ratio seen at the first visit appears to be affected by higher, albeit not statistically significant, PlGF levels in the GDM group. This reflects similar to previous work which has shown PlGF levels to be higher in early pregnancies that are either affected by pre-existing non-insulin requiring T2DM or who go on to develop GDM (Ong et al., 2000). This is of interest, as it is the opposite to what is seen in women who present with suspected preeclampsia. Lower levels of PlGF, and higher sFlt1:PlGF are associated with placental vascular maladaptation and subsequent preeclampsia development (Kleinrouweler et al., 2012). Recent landmark trials in the use of PlGF and the sFlt1: PLGF based assays, have enabled more widespread use of these biomarkers to help rule out current or impending preeclampsia in women with suspected symptoms in clinical guidelines (Chappell et al., 2013, Duhig et al., 2019, PlGF-based testing to help diagnose preeclampsia (National Institute for Health and Care Excellence), 2016), but low specificity for longer term (>14 days) or asymptomatic prediction of preeclampsia has prevented more widespread adoption (Kleinrouweler et al., 2012). These large trials included women with pregestational and gestational diabetes, but these women comprised the minority of the study populations, as would be expected in the general obstetric population. Given the differences demonstrated in this study and in others around PlGF in diabetes, it would seem pertinent to explore whether these PlGF based tests hold the same predictive value in the population with diabetes.

In the same study by Ong et al, similar results of higher PlGF were not evident in the subgroup with insulin-requiring diabetes suggesting that PlGF effects in diabetic pregnancy are not mediated predominantly by hyperglycaemia (Ong et al., 2000). Our data support this with no correlation seen between glucose and

PlGF. Ong et al hypothesised that insulin resistance may be the pathophysiological mechanism driving higher PlGF, but there was no significant correlation with PlGF and HOMA-IR or insulin concentration values in my study. The results are interesting as it seems to suggest differences in placental development and function across the spectrum of diabetes phenotypes. Interestingly, research in non-pregnant subjects has shown elevated PlGF to be an antecedent risk factor for development of future incident T2DM and is also independent of the traditional markers of glycaemia (HbA1c) and insulin resistance.

Inflammatory cytokine pathways are upregulated in metabolic disease and thought to contribute to preeclampsia risk (Rusterholz et al., 2007, B et al., 2011, Lau et al., 2013). The original methods of this study, therefore also included measurement of some of the most commonly implicated inflammatory cytokines (TNF-α, IL-6, IL-1ß) to try identify any association with these biomarkers and maternal endothelial function in GDM. However, concerns regarding the assay results and limited laboratory time for repetition of experiments meant that this aspect of my study was excluded from ongoing analysis.

In conclusion, endothelium-dependent macrovascular vasodilatation is similar in women with GDM compared to pregnant women without diabetes. Macrovascular endothelial dysfunction is unlikely to be a major contributory mechanism to the increased risk of adverse outcomes seen in GDM pregnancy. Higher glycaemia is however significantly associated with higher maternal BP suggesting vascular dysfunction independent of endothelial function occurs. Further work into microvascular endothelial function and non-endothelial vascular pathways is warranted to better understand pathophysiology in such pregnancies. An unexpected secondary outcome of this work was of lower sFlt-1:PlGF and higher PlGF in GDM pregnancy, raising suspicions that current PlGF cut-offs used in preeclampsia diagnosis may not hold relevance to the obstetric population with diabetes. Ongoing work exploring their accuracy in this population will be important given the widespread use of such assays in clinical practice.

6 Chapter 6: Placental vascular function in gestational diabetes versus non-diabetic controls

6.1 Introduction

My data, and data from others have shown that pregnancies complicated by diabetes have significantly increased risk of adverse outcomes, with up to 6-fold higher rates of preterm delivery and 5-fold higher rates of stillbirth than the background obstetric population (Mackin et al., 2019, Murphy et al., 2021). Many preterm deliveries will occur due to concerns of preeclampsia or stillbirth. As I have already discussed, diabetes increases preeclampsia risk, particularly in pregestational diabetes where rates are 4-fold higher (Duley, 2009), but it also occurs with higher frequency in gestational diabetes (GDM) (Weissgerber and Mudd, 2015). The pathophysiology of preeclampsia has not been fully elucidated, but is recognised to be placental in origin with high resistance uteroplacental circulation and vascular dysfunction. The details of this are discussed more in chapter 1.2.2 (Raymond and Peterson, 2011). Fetal growth may be compromised because of reduced placental perfusion, and in some cases can be severe enough to cause intrauterine death. In diabetes, there appears to be a dynamic relationship between maternal glycaemic status and preeclampsia risk, even in the later stages of pregnancy, beyond the stages of placental vascular development (Metzger et al., 2008). Third trimester maternal HbA1c in pregestational diabetes is associated with risk of preeclampsia over and above first trimester HbA1c and the risk associated with coexisting diabetes complications (Holmes et al., 2011). In gestational diabetes (GDM) reduction of glucose reduces risk of preeclampsia despite this intervention only starting after 24-28 weeks and reflecting a much milder biochemical abnormality than in pregestational diabetes (Landon et al., 2009, Crowther et al., 2005). Taken together, this suggests that glycaemia is an important modifiable risk factor for preeclampsia and importantly appears to have effects that occur beyond first trimester placental development. However, the placenta remains central to preeclampsia pathogenesis in diabetes, with the only recognised curative treatment being delivery of the placenta. It is also of interest that even in the

absence of preeclampsia women with diabetes have a high prevalence of subclinical placental vascular insufficiency detected on histopathological examination of placentae (Daskalakis et al., 2008). This leads us onto the hypothesis that placental vascular function is adversely affected by diabetes, and may be important in the pathophysiology of adverse outcomes seen in such pregnancies.

Alterations in placental vascular function have been reported in patients with adverse outcome pregnancy. Chorionic plate vessels are those vessels which control oxygen and nutrient delivery from placenta to the fetus and are important to regulate fetal growth. Chorionic plate veins deliver nutrients to the fetus, whilst the chorionic plate arteries (CPAs) return deoxygenated blood and toxins to the placenta. CPAs appear to be integral in regulating placental vascular resistance. Wire myography studies have shown reduced vasoconstrictive and vasodilatory responses of the chorionic plate arteries in preeclamptic pregnancy compared with uncomplicated pregnancy (Ong et al., 2002). Similar changes are also seen in placental vessels of growth restricted pregnancies suggesting compromise of fetal-placental perfusion as a cause for adverse neonatal outcomes (Mills et al., 2005). However, most mechanistic studies of the chorionic plate actively exclude women with diabetes, leaving a paucity of data in this high-risk group. I explored whether fetoplacental vascular function is altered in GDM, and generate further hypothesis about how this may relate to adverse pregnancy outcomes.

6.2 Methods

Placentas were collected prospectively from the same cohort recruited as part of the wider study described in earlier chapters, and split into GDM and non-GDM groups.

Following alert from the clinical team, the principal investigator (Dr Sharon Mackin) collected placentas immediately following delivery and transported them to the lab where wire myography experiments were conducted on CPAs.

Experimental conditions are described in detail in chapter 2.3. In brief, CPAs were mounted on wire myographs and normalised to $0.9L_{5.1kPa}$ whilst bubbled with $5\%0_2/5\%CO_2/N_2$.

Vessel viability was confirmed by measuring contractile response to 62.5 mmol K^+ containing salt solution (KPSS). Cumulative dose response curves were then assessed to $1x10^{-10}$-$3x10^{-5}$M U46619 to assess contractility. Vasodilatory response was then assessed to either:

- **Bradykinin ($1x10^{-10}$ mol - $3x10^{-5}$ mol)** – endothelium-dependent vasodilator

- **Sodium Nitroprusside (SNP) ($1x10^{-10}$ mol -$3x10^{-4}$mol)** – endothelium-independent vasodilator

- **Calcitonin-gene related peptide (CGRP) ($1x10^{-10}$mol -$3x10^{-4}$ mol)** – endothelium-dependent and -independent vasodilator actions

- **Calcitonin-gene related peptide (CGRP) ($1x10^{-10}$ mol -$3x10^{-4}$ mol) + 20-minute incubation with the 100 µmol of nitric oxide synthase (NOS) inhibitor, N(ω)-nitro-L-arginine methyl ester (L- NAME)** to determine if acting in endothelium-dependent or -independent manner in CPAs

Maximal vasoconstrictive responses are expressed as % vasoconstriction relative to maximum tension reached following 62.5 mmol KPSS. Vasodilatory responses are expressed as % vasodilation relative to the maximum tension reached following preconstriction with $3x10^{-7}$ mol U46619.

6.2.1 Statistical analysis

Statistical analysis was performed using IBM SPSS™ statistics v 22.0 and Graphpad Prism™ v 8.0 (GraphPad Software Inc™, USA). Raw vessel tension data was analysed using LabChart Reader™ software v 8.1.13 (AD Instruments™, UK).

Maternal and fetal demographics between groups were compared using one way ANOVA or χ^2 as appropriate. For each CCRC, comparison of curves between groups was compared using extra sum of squares F test. Differences in mean Emax and mean EC_{50} for each agonist were compared between groups using one-way ANOVA. Statistical significance was assumed if $p<0.05$.

6.3 Results

In total, 32 placentas from the original cohort of 60 were women obtained. This included 16 women with GDM, 9 women from the risk factor group and 7 women from the control group. The remainder (n=28) were not obtained due to variable reasons of: researcher unable to collect immediately or researcher not contacted by clinical team (n=22), home birth (n=1), researcher unavailable to perform myography (n=2), non-viable vessels for experimentation (n=2) and a clinical need for immediate transfer of placenta to NHS pathology services (n=1).

6.3.1 Maternal and delivery characteristics of study population

Maternal and delivery characteristics of those women who had placentas included in myography studies is detailed in table 6.1. The key differences between groups are similar to those seen in the larger cohort, with GDM mothers having higher BMI than control women but similar to the risk factor group and GDM mothers delivering at earlier gestation. There was no statistical difference in mode of delivery between groups, but numbers are too small to detect this with adequate power. There was no difference in mean infant birthweight across groups.

Table 6.1: Maternal and delivery characteristics of woman with placentas included in myography studies (n=32)

	GDM (n=16)	Risk factor (n=9)	Control (n=7)
Mean maternal age, years (SD)	32.0 (5.6)	31.3 (5.0)	32.7 (6.5)
Booking BMI	32.6 (6.9)**	31.3 (7.0)*	24.1 (3.7)
Last measured mean maternal SBP pre-delivery, mmHg (SD)[a]	105 (14)	110 (20)	99 (16)
Last measured mean maternal DBP, mmHg pre-delivery (SD)[a]	66 (11)	72 (16)	64 (8)
Mean gestational age at delivery, weeks (SD)	38.4 (1.0)ǂǂ	40.4 (1.2)*	39.3 (0.6)
Male infant (%)	44% (n=7)	44% (n=4)	0
Mean birthweight, g (SD)	3426.6 (643.1)	3387.1 (492.1)	3426.1 (179.3)
Mean GROW™ birthweight centile (SD)	59.0 (29.3)	48.4 (34.2)	60.9 (33.5)
Mean placental weight, g (SD)	504.6 (148.3)	473.9 (119.7)	499.0 (91.2)
Method of delivery			
Vaginal delivery	56% (n=9)	44% (n=4)	71% (n=5)
Elective caesarean	31% (n=5)	44% (n=4)	29% (n=2)
Emergency caesarean	13% (n=2)	12% (n=1)	0

*p<0.05, ** p<0.01 versus controls by one way ANOVA or χ^2 as appropriate

ǂǂ p<0.001 versus risk group by one way ANOVA or χ^2 as appropriate

[a] Last measured BP pre-delivery was at visit 4 in 31 cases and visit 3 in 1 case

6.3.2 Vasocontraction to U46619 (thromboxane A2 mimetic)

In total, 144 viable vessels from 32 placentas were examined. This included 71 vessels from 16 GDM placentas, 29 vessels from 9 risk factor placentas and 19 vessels from 7 control placentas.

Mean vessel diameter were 310 (69), 278 (49) and 382 (112) μm for GDM, risk factor and control vessels respectively (p=0.2).

The maximum response to 62.5 mmol KPSS max (absolute tension) was similar at 2.6 mN GDM, 2.0 mN risk factor and 2.4 mN in the control group. There were no differences in vessel contractility to U46619 across groups (Figure 6.1A). Mean Emax was similar across groups (167.1% control, 161.1% risk factor and 149.9 GDM), as was $logEC_{50}$ (-7.04 mol control, -7.01 mol risk factor and -7.08 mol GDM). Individual data points are shown in figures 6.1B and 6.1C).

6.3.3 Vasodilatation to bradykinin (endothelium-dependent)

19 viable CPAs from 6 placentas (5 risk factor, 1 GDM) were pre-constricted with $3x10^{-7}$ mol U46619, followed by exposure to cumulative concentrations of bradykinin ($1x10^{-10}$ mol – $3x10^{-5}$ mol). There was no significant vasodilatory response of CPAs to bradykinin (figure 6.2). Bradykinin was removed from the myography protocol at this stage.

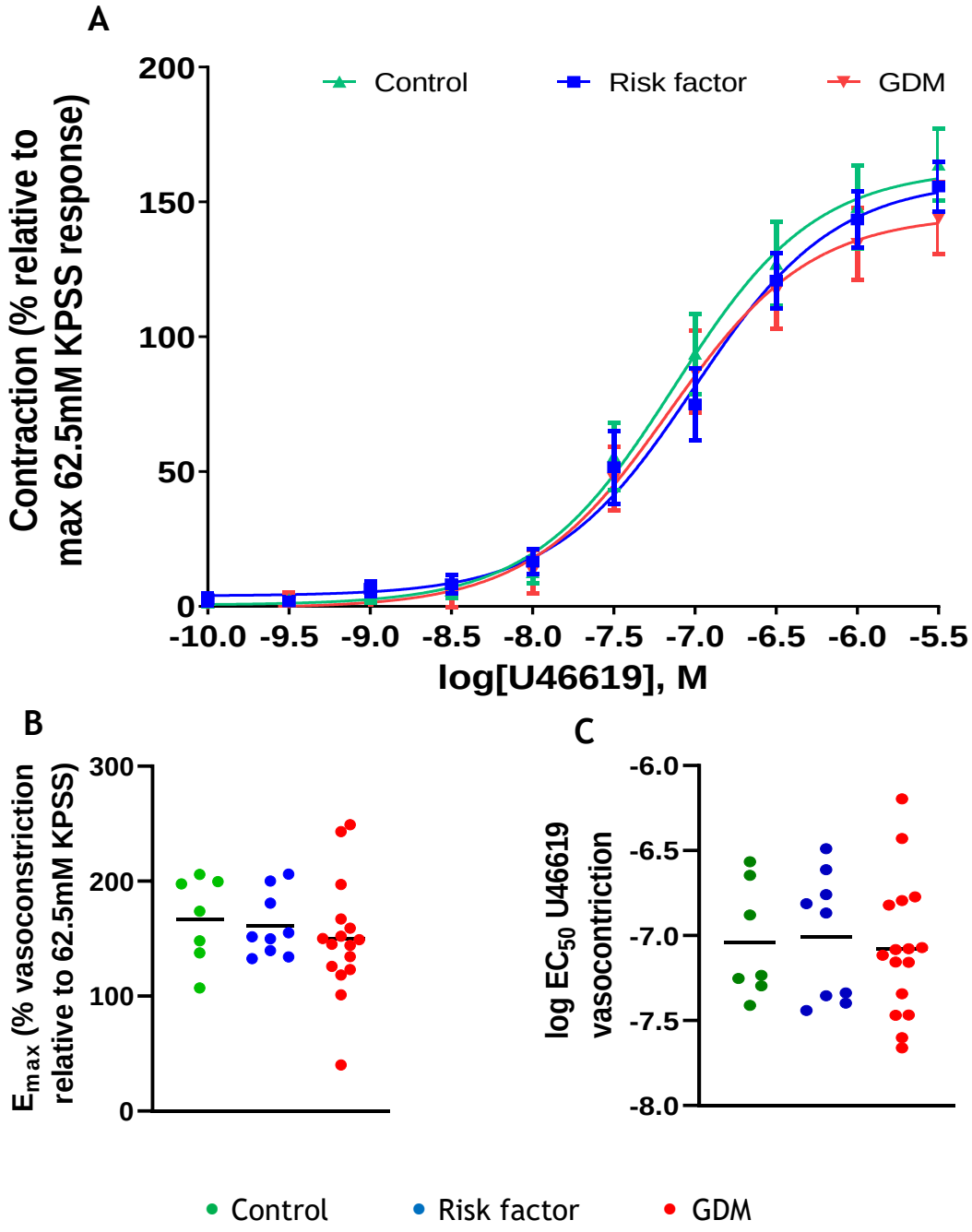


Figure 6.1: Vasoconstrictive response of CPAs to U46619 from women with GDM, risk factors for GDM but normoglycaemia and healthy controls

There was no difference in contractility of CPAs taken from placentas from women with GDM, risk factors for GDM or healthy controls (A). There was no difference in the maximal contractile response to U46619 (B), nor in the log EC_{50} between groups (C). GDM groups are shown in red, risk factor group in blue and control group in green.

The cumulative concentration response curve shows mean + SEM, and was compared using the extra sum of squares F test. The scatter plot diagrams show mean E_{max} and EC_{50} for individual subjects, with a group mean demonstrated by the line. Where more than one vessel was used for a subject, the average of the readings across the vessels for that subject was used. The mean E_{max} and EC_{50} between groups was compared using one way ANOVA (p n.s. for all).

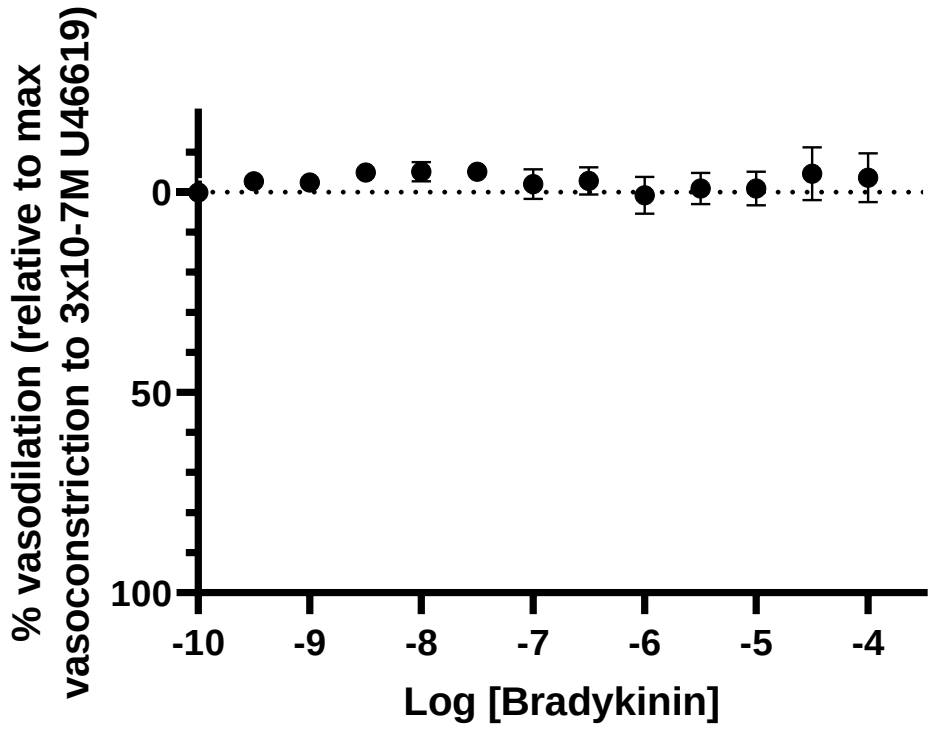


Figure 6.2: Vasodilatory response of CPAs to increasing concentrations of Bradykinin

There was no demonstrable vasodilatation of CPAs to bradykinin after pre-constriction with $3x10^{-7}$M U46619. This analysis included 19 vessels from 6 subjects. Each dot represents the mean % vasodilatation at each log[bradykinin] whilst the error bars show SEM.

6.3.4 Vasodilatation to CGRP

Following preconstriction with $3x10^{-7}$ mol U46619, CGRP vasodilatory CCRC was conducted for 43 CPAs from 23 placentas. This included 25 vessels from 14 GDM placentas, 5 vessels from 3 risk factor group placentas and 13 vessels from 6 control placentas. Due to the low number of subjects included in the risk factor group, analysis of this CCRC was restricted to GDM versus non-GDM groups.

Vessels were of similar mean diameter at 315±66μm and 300±99μm in GDM and non-GDM groups respectively. There was no difference in CPA vasodilatory response to CGRP between GDM and non-GDM pregnancy (p=0.6, figure 6.3A). $LogEC_{50}$ (-7.9 mol both groups) and E_{max} (35.2±23.8% GDM, 30.8+16.3% non-GDM) were similar between groups. Individual data points for both are shown in figures 6.3B and 6.3C.

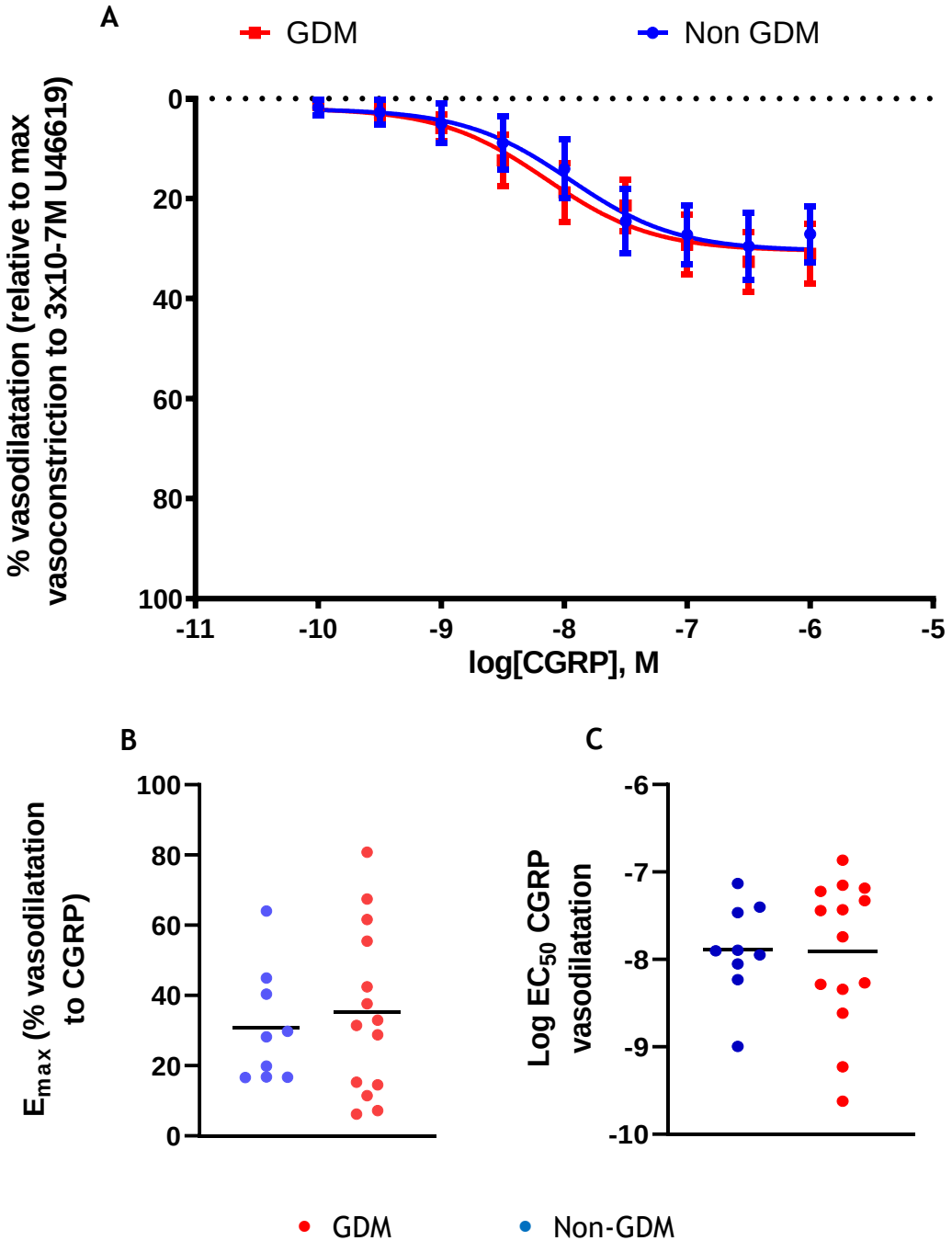


Figure 6.3: Vasodilatory response of CPAs to CGRP from women with and without GDM

Following preconstriction with $3x10^{-7}$M U46619, there was no difference in vasodilatation response of CPAs to CGRP in placentas from women with GDM and those without GDM (A). There was no difference in the mean maximal vasodilatory response to U46619 (B), nor in the log EC_{50} between groups (C). GDM groups are shown in red and non-GDM group in blue.

The cumulative concentration response curve shows mean $\pm$ SEM, and was compared using the extra sum of squares F test. The scatter plot diagrams show mean E_{max} and EC_{50} for individual subjects, with a group mean demonstrated by the line. Where more than one vessel was used for a subject, the average of the readings across the vessels for that subject was used. The mean E_{max} and EC_{50} between groups was compared using student t-test (p n.s. for all).

6.3.4.1 Vasodilatory response of CPAs to CGRP in presence of NOS inhibition

There were 7 placentas (from entire cohort) with sufficient viable vessels to allow simultaneous CGRP CCRCs to be conducted – one cohort as described above, and others following the same protocol but after a 20-minute incubation with 100μM L-NAME. Figure 6.4 shows that there was no difference in vasodilatory response of CPAs to CGRP in the presence of NOS inhibition, suggesting that CGRP effects on CPAs occur predominantly via endothelium-independent mechanisms.

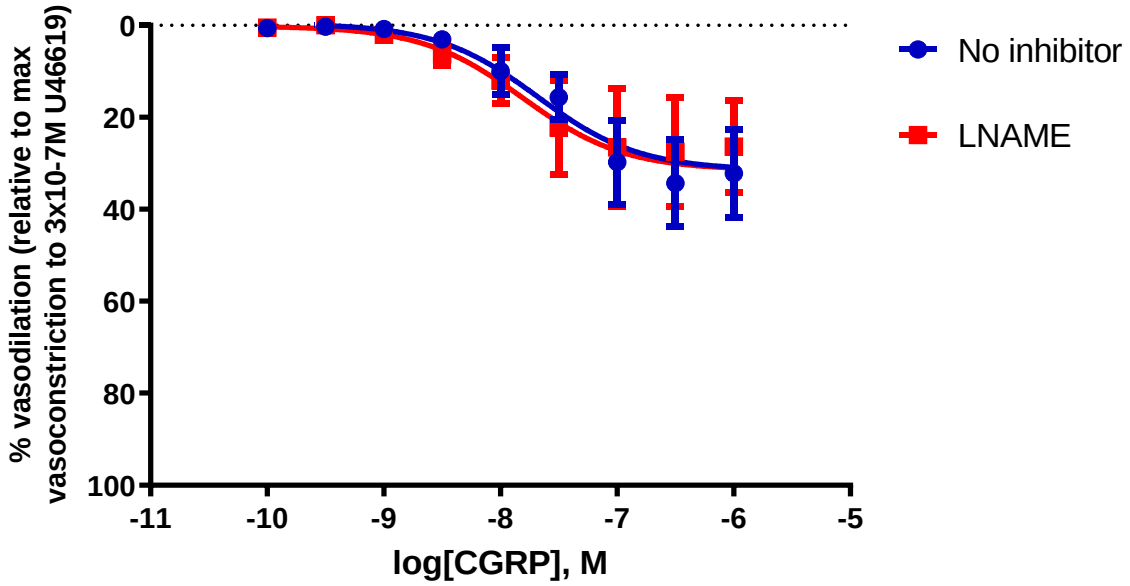


Figure 6.4: Vasodilatory response of CPAs with and without NOS inhibition

There was no difference in vasodilatory response of preconstricted CPAs to CGRP ($1x10^{-10}$M to $3x10^{-4}$ mol) following incubation with the NOS inhibitor L-NAME (100 μM) compared to non-incubated vessels. This suggests that CGRP effects on CPA vasodilatation occur mainly through endothelium-independent pathways.

Those vessels incubated with L-NAME (12 vessels from 7 placentas) are shown in red, whilst those without prior incubation (10 vessels from 7 placentas) are shown in blue. The best fit cumulative concentration response curve shows mean ± SEM, and was compared using extra sum of squares F-test.

6.3.5 Vasodilatation to SNP (endothelium-independent)

Endothelium-independent vasodilatation response were assessed with increasing concentrations of SNP ($1x10^{-10}$ mol -$3x10^{-4}$ mol). This cohort included 15 vessels from 9 GDM placentas, 13 vessels from 6 risk factor placentas and 11 vessels from 6 control placentas.

Mean vessel diameters were similar across groups at 341±120μm for GDM, 298±103μm for risk factor group and 364±145μm for control group (p=0.4).

Vessels from GDM placentas had a significantly attenuated vasodilatory response to cumulative doses of SNP, compared with vessels from the risk factor and control cohorts ($p<0.0001$ for maximum vasodilatation using best fit curve analysis, see figure 6.5A). There appeared to be greater variation amongst mean E_{max} in individual subjects in the GDM group (see figure 6.5B). Mean E_{max} was 49.5±11.0%, 73.9±11.0% and 74.0±12.0% in GDM, risk-factor and control groups respectively (p=0.05).

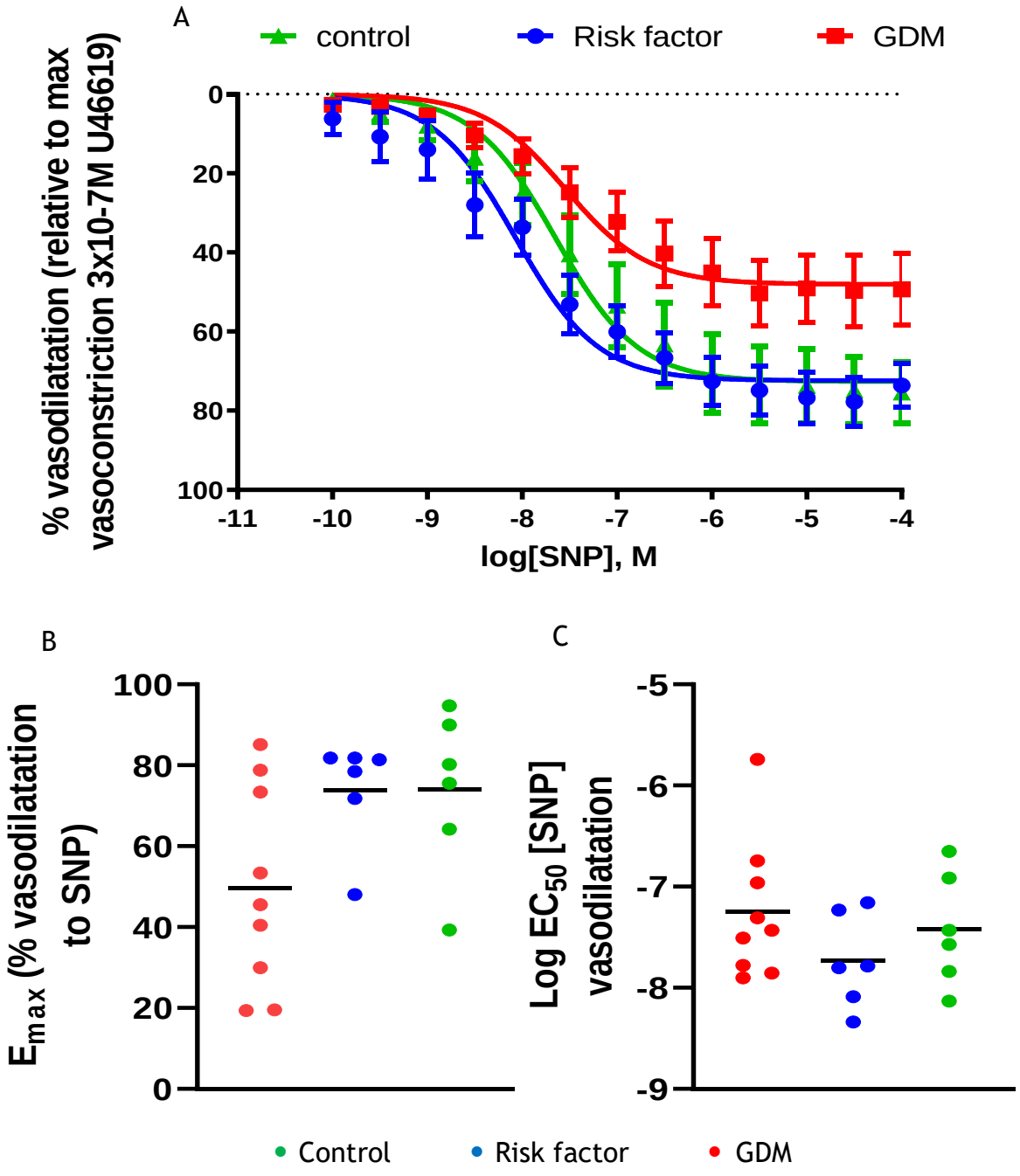


Figure 6.5: Vasodilatory response of CPAs to SNP from women with GDM, risk factors for GDM but normoglycemia and healthy controls

Following pre-constriction with $3x10^{-7}$M U46619, CPAs from women with GDM had attenuated vasodilatation to increasing concentrations of SNP ($1x10^{-10}$-$3x10^{-4}$mol). Best fit cumulative concentration curves were fitted and compared using extra sum of squares F test, as shown in figure A ($p<0.001$ for maximal response different across groups). Dots represent the mean response whilst error bars show SEM.

CCRCs were then fitted for each subject, and mean Emax and EC_{50} data for each individual subject are shown in figure B and C accordingly. Mean Emax was 49.5+11.0% in GDM compared to 73.9+11.0% and 74.0+12.0% in the risk factor and control groups ($p=0.05$ one way ANOVA).

6.3.5.1 Effect of fasting glucose and insulin on SNP vasodilatation

There was a significant inverse correlation of fasting plasma glucose measured at visit 4 with E_{max} to SNP (Pearson's r -0.526, p=0.01, figure 6.6). The effect of glucose on E_{max} remained significant when tested in a general linear model that included diabetes diagnosis, fasting glucose and their interaction (p=0.03).

Insulin resistance, as measured by HOMA-IR was also negatively correlated with Emax to SNP (Pearson's r -0.521, p=0.02, figure 6.7). When this was tested in a model that included diagnosis, HOMA-IR and its interaction, the effect of HOMA-IR on was attenuated (p=0.08).

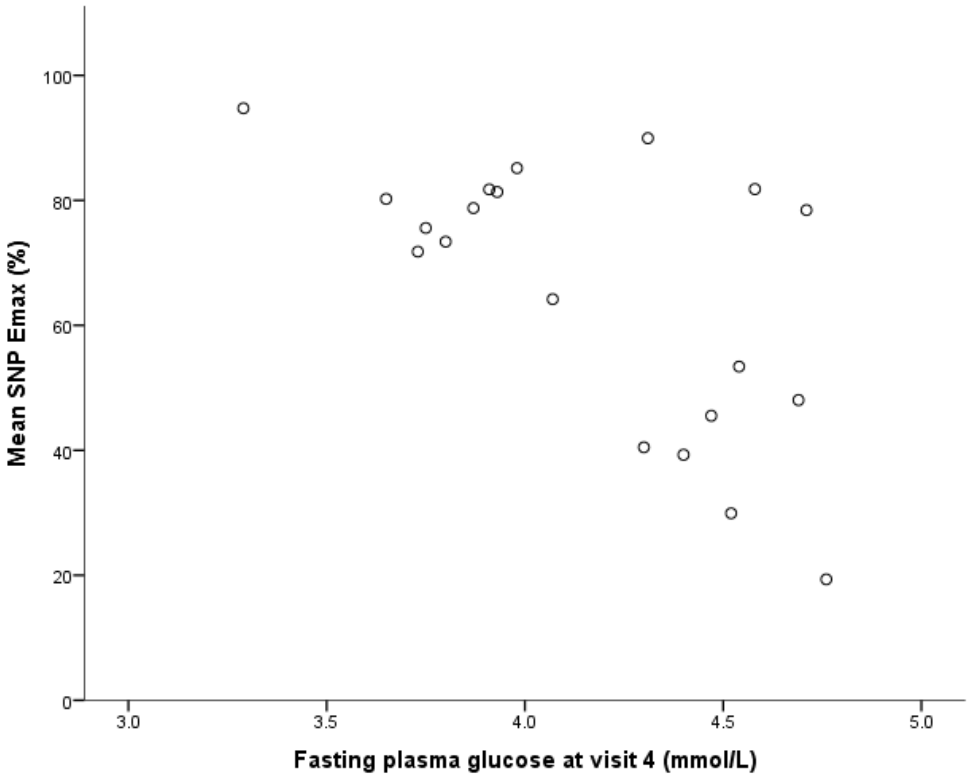


Figure 6.6 Scatterplot diagram showing relationship of fasting plasma glucose at end of pregnancy to E_{max} of SNP for CPAs

This scatterplot shows a moderate negative correlation with fasting plasma glucose (x axis) measured at visit 4 (approximately 36 weeks gestation) and the maximal vasodilatory response of CPAs to the endothelium independent vasodilator, SNP (y axis) (Pearson's r -0.526, p=0.01).

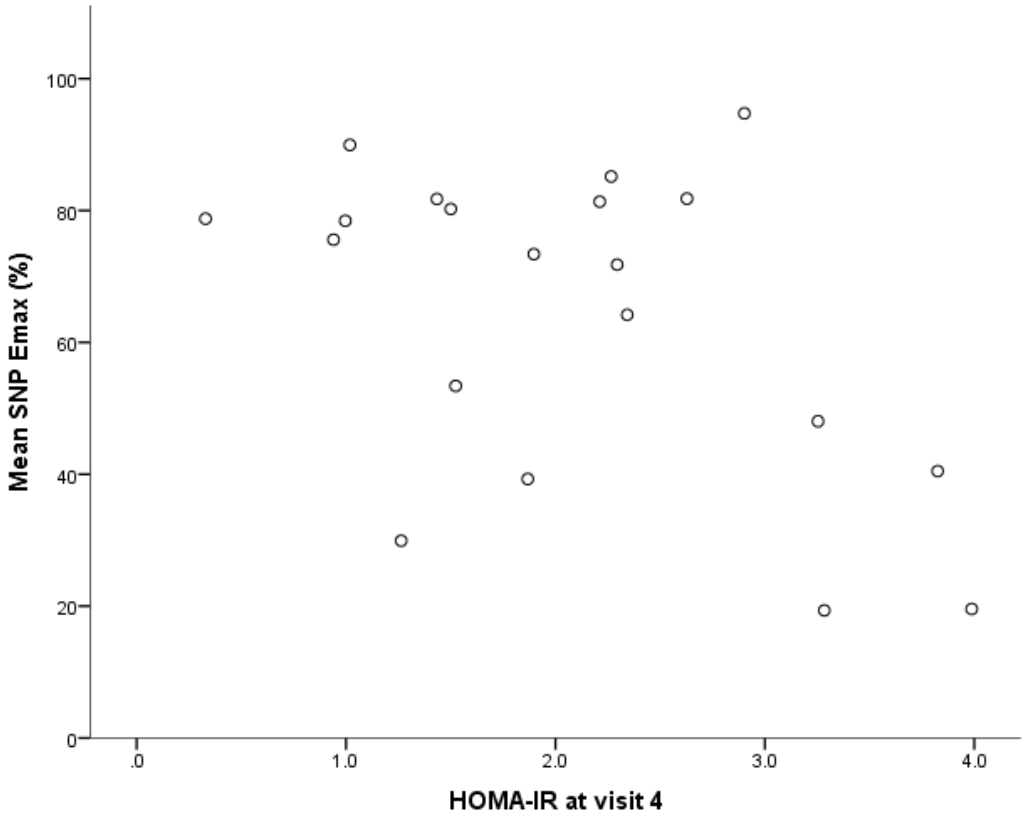


Figure 6.7: Scatterplot diagram showing relationship of insulin resistance scores (HOMA-IR) at end of pregnancy to E_{max} of SNP for CPAs

This scatterplot shows a similar negative correlation with insulin resistance, as measured by calculated HOMA-IR (x axis) from fasting bloods taken at visit 4 (approximately 36 weeks gestation) and the maximal vasodilatory response of CPAs to the endothelium independent vasodilator, SNP (y axis) (Pearson's r -0.521, p=0.02).

6.3.5.2 Effect of circulating maternal angiogenic factors on SNP vasodilatation

There was no correlation between Emax to SNP and concentrations of sFlt-1, PlGF nor the sFlt-s:PlGF ratio found in maternal blood. Numbers available for analysis were small (n=15 for sFlt-1, 13 for PlGF and 8 for sFlt-1:PlGF), the reasons for which are detailed in chapter 5.2.

6.3.5.3 Effect of delivery method on SNP vasodilatation

Although not statistically significant, there were observed differences in the number of women undergoing caesarean and vaginal delivery between groups. Subgroup analysis is limited by small numbers and so to assess whether these methods of delivery had statistical impact on the mean SNP Emax observed

between groups, a general linear model that incorporated both diagnosis, delivery method (vaginal versus caesarean delivery) and the interaction between the two was utilised. Only diagnosis had a significant effect on SNP Emax (p=0.07 for diagnosis) whilst delivery method and interaction between the two remained non-significant (p=0.11 and 0.9 respectively).

Similarly, the effects of drugs used during delivery such as prostin and syntocinon were analysed to see if significant effect on vasodilatory response to SNP. In total, 7 of the 21 women received prostin for labour induction (1 risk factor, 6 GDM women), and all women received syntocinon whether that be for induction/augmentation of labour or routine use in the third stage of labour.

In a generalised linear model that incorporated terms for diagnosis, prostin use and the interaction between the two, prostin use did not have a significant effect on Emax (p=0.4), although effect of diagnosis was augmented (p=0.1). Syntometrine was used to manage the third stage of labour in 5 women (3 GDM, 1 risk factor and 1 control subject) and in a similar model, did not significantly alter Emax (p=0.3). Syntocinon effect was not analysed as it was used in all women.

6.3.5.4 SNP CCRC and relationship to birthweight centile

For those women who had vessels included in SNP CCRC analysis, birthweight data are shown in table 6.2. Absolute birthweight (grams) and birthweight GROW centile were similar across groups. In a generalised linear model that included SNP Emax and diagnosis, birthweight centile was not significantly affected by either.

Table 6.2: Mean birthweight (g) and birthweight GROW centile according to diabetes diagnosis for women included in SNP CCRC analysis

	Mean birthweight, g (SD)	Mean birthweight GROW centile (SD)
GDM (n=9)	3467 (312)	61.1 (19.3)
Risk factor (n=6)	3646 (710)	54.6 (38.00
Control (n=6)	3363 (487)	57.1 (34.9)

No statistical difference between groups using ANOVA.

6.3.6 Discussion

In this study, CPAs from GDM pregnancies have reduced endothelium-independent vasodilatory capacity, suggesting a pro-contractile phenotype of placental vasculature. These differences have been demonstrated in the fetal side of the placental circulation, which could have direct and detrimental effects on fetal growth and development (Mills et al., 2005). Importantly, differences were not seen in the group of mothers with risk factors for diabetes but normal glucose tolerance, suggesting that these effects are specifically diabetes, and not obesity related. This is further corroborated by our data showing an association with higher maternal glucose and lower vasodilatory response to SNP.

To my knowledge, this is the first study to explore fetoplacental vascular function in GDM pregnancy, and there is a paucity of placental studies in pre-existing diabetes. A study using dual-perfused placental cotyledon methodology showed attenuated endothelium-independent vasodilatory response to glyceryl trinitrate in T1DM compared to non-diabetes controls (Kossenjans et al., 2000). This study also showed diminished responses in preeclamptic pregnancies. Similar to SNP, glyceryl trinitrate has similar pharmacological properties whereby it acts through NO donation to vessels to cause vasodilation. Diminished vasodilatory capacity of fetoplacental vessels to SNP has also been associated with preeclampsia (Ong et al., 2002). CPAs have a key contribution to maintain placental vascular resistance. Taking this together with our findings,

we hypothesise further that there is higher vascular resistance in the fetoplacental unit in GDM pregnancy, which could compromise fetal nutrient transfer and perfusion. Although speculative, this may be an important mechanism for increased adverse fetal outcomes. In preeclampsia increased placental vascular resistance is also thought to contribute to dysregulated production of placental growth factors and inflammatory markers which when released into maternal circulation, cause widespread endothelial dysfunction and the clinical syndrome of preeclampsia (Tomimatsu et al., 2017). Reduced capacity to vasodilate in GDM could contribute to increased resistance on the fetal side of the placental circulation, which could lead to fetal hypoxia and stimulation of angiogenic and inflammatory cytokine production similar to what is seen in preeclampsia. The measured angiogenic markers (sFlt-1 and PlGF) did not correlate with maximum vasodilatory response in my study, but were limited by small numbers of placentas examined and furthermore by lack of reliable sFlt-1/PlGF results across the group. There are also several inflammatory and anti-angiogenic mediators implicated in preeclampsia pathophysiology that are unmeasured in this study.

Interestingly, there was no difference in vasodilation of CPAs to CGRP between groups. CGRP receptors are expressed abundantly in placental endothelial, VSMCs and trophoblast cells (Dong et al., 2003, Dong et al., 2004). Both CGRP receptor expression and concentrations of CGRP in maternal and cord blood are reduced in pregnancies complicated by preeclampsia (Dong et al., 2005, Dong et al., 2006). More interestingly, others have shown CGRP concentrations in fetal and cord blood correlate with fetal growth (Parida et al., 1998). This is suggesting an important role of CGRP in maintaining placental vascular flow to support fetal growth. CGRP also bears close homology to other proteins (adrenomedullin, amylin) that have physiological effects on glycaemic control. These hormones in addition to CGRP have been shown to be dysregulated in diabetes and insulin resistance, and can contribute to oxidative stress (Brain and Grant, 2004, Shimosawa et al., 2003). These features made CGRP a novel agonist to explore further in GDM pregnancy.

The present studies showed that CGRP exerted vasodilatory effects predominantly through endothelium-independent mechanisms in placental vessels. The endothelium-independent pathways through which CGRP affects VSMCs are different to those through which SNP acts. Briefly, CGRP acts via G-protein coupled receptors to activate cAMP and intracellular calcium pathways within VSMCs, whilst SNP donates NO to activate cGMP pathways (see figure 6.8). Since there is significant difference between groups in SNP induced vasodilation but not CGRP, we can conclude that placentas from GDM pregnancy have direct or indirect dysregulation of VSMC cGMP pathways.

There was no significant vasodilatory response to the endothelium-dependent agonist, bradykinin. This has been demonstrated by others also (Mccarthy et al., 1994, Ong et al., 2002). This is despite presence of appropriate receptors for agonists present in these tissues (Valdés et al., 2016). Potential reasons for such findings include high levels of peptidases in placental tissue that could metabolise peptide agonists, or reduced intraluminal contact with agonists during wire myography experiments may not be sufficient to mimic in vivo conditions. Hayward et al showed that addition of high dose Bradykinin to pre-constricted CPAs reduced perfusion pressure in these vessels, but not in the same measurable way used in standard myography techniques where there is progressive vasodilation with increasing agonist concentration. Instead, there was reduced amplitude and frequency of vasomotor oscillations (Hayward et al., 2013). In constricted CPAs, vasomotion is slow and can occur as little as once every 15 minutes (Hayward et al, 2013), or may not be induced in some vessels at all as I learned through my experience in the specialist unit at the University of Manchester. Vasomotion is observed in many vascular beds and may serve a role in optimising fetoplacental flow, but this role is less clearly defined. Furthermore, others have shown marked vasodilation in response to bradykinin in pressure myography studies (Gray et al., 2015). Taken together with previous evidence that flow-induced NO release is present in fetoplacental circulation and histopathological evidence of bradykinin receptors in these tissues, it seems likely that endothelial induced vasodilation is an important mechanism in

regulating fetoplacental flow but that wire myography methodologies are very limited in the ability to demonstrate this.

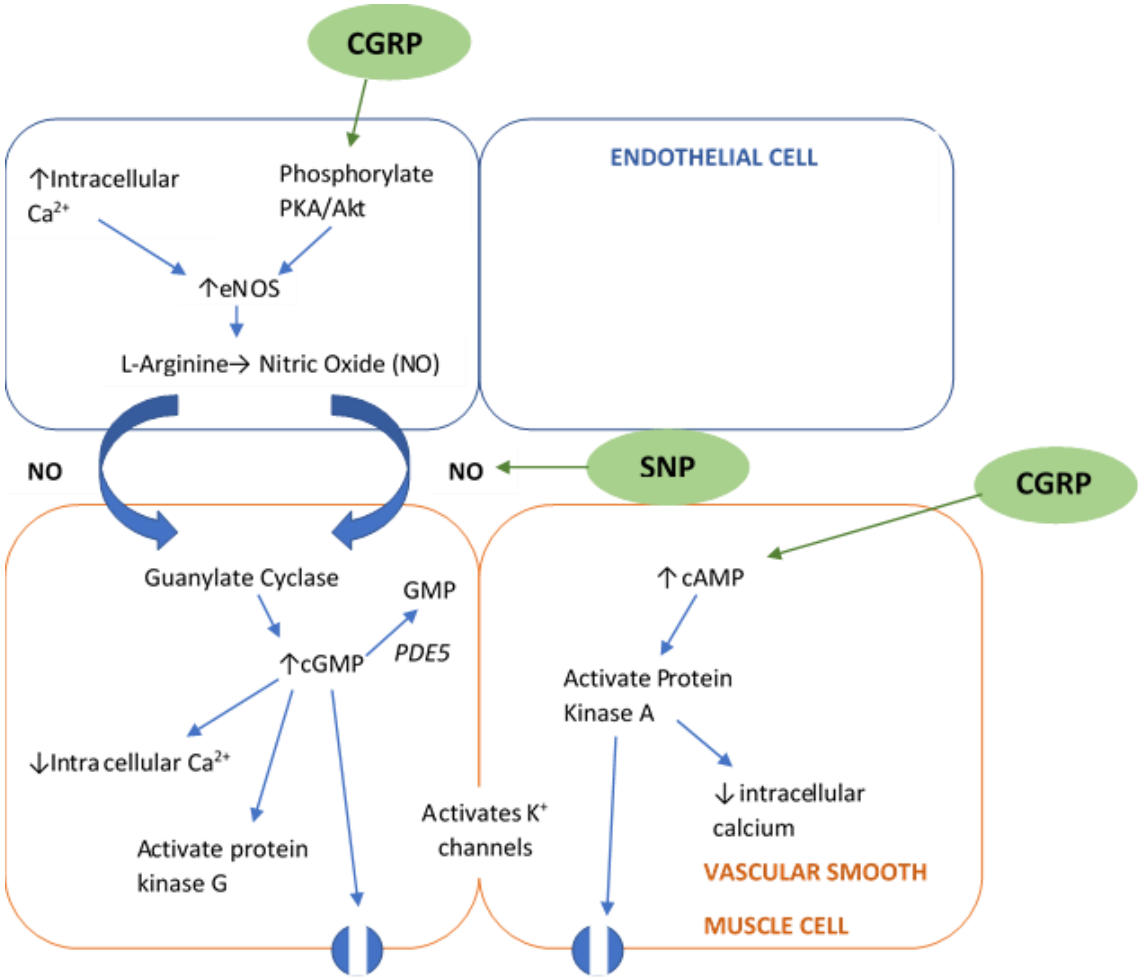


Figure 6.8: Endothelial and vascular smooth muscle pathways involved in vasodilatation and the effects of CGRP and SNP

This figure shows the endothelial cell in blue and VSMC in orange. Within the endothelial cell, nitric oxide (NO) is produced from the oxidisation of L-Arginine by the catalyst endothelial Nitric Oxide Synthase (eNOS). eNOS can be stimulated through pathways which increase intracellular calcium production within the endothelial cell, or via calcium independent pathways that increase cAMP and phosphorylation of protein kinases (PKA and Akt). Following synthesis, NO diffuses across the endothelium into the VSMC where it acts to increase levels of the second messenger, cGMP. cGMP pathways cause vasodilatation through several mechanisms. These include 1) reduction in intracellular calcium stores by inhibiting release from the endoplasmic reticulum 2) activation of voltage gated K channels which cause a K influx and repolarisation of the VSMC and 3) activation of protein kinase G which activates myosin phosphatase resulting in dephosphorylation of myosin light chains and inhibiting vasoconstriction (Gewaltig and Kojda, 2002). cGMP is deactivated by Phosphodiesterase 5 (PDE5).

VSMC have NO independent mechanisms for vasodilatation. When cAMP pathways are activated by agonists, they cause phosphorylation and activation of protein kinase A. This in turn leads to activation of potassium gated channels on the cell membrane, activated myosin phosphatase with reduced calcium sensitivity of contractile apparatus and increased calcium uptake by the endoplasmic reticulum. All of this results in vasodilatation (Consigny, 1991). cAMP is broken down by Phosphodiesterase 3.

In this study, NO donation via SNP was used and would activate cGMP pathways. CGRP activates cAMP within both the endothelial and VSMC cell, with subsequent phosphorylation of protein kinases as described.

The mechanisms by which GDM may alter cGMP pathways in placental VSMCs needs further exploration. One hypothesis is that expression of phosphodiesterases (PDE), is upregulated in hyperglycaemic pregnancy. PDEs hydrolyse cGMP and cAMP and inactivate it causing smooth muscle contraction. PDE5 is cGMP specific and expressed in placental vasculature (Larré et al., 2017). Plasma PDE5 and PDE activity within the placenta has been found to be increased in women with preeclampsia compared to healthy controls (Pinheiro Da Costa et al., 2006, Sohlberg et al., 2014). Hyperglycaemia has also been shown to increase PDE5 expression in neurovascular tissues in non-pregnant mouse models (Wang et al., 2011). It seems plausible that similar altered expression may occur in the placenta of diabetic pregnancy as an effect of hyperglycaemia. However, further studies are needed, initially exploring the expression of cGMP and PDE5 in these placentas before definitive conclusions can be drawn.

It is also likely that increased oxidative stress from reactive oxygen species (ROS) plays a role in the altered vascular smooth muscle function seen here. Superoxide (O_2^-) is the most abundant ROS in humans and reacts with NO to produce the cytotoxic peroxynitrite ($ONOO^-$). ROS dysregulation has been implicated in many vascular diseases, and in health is kept in check by ROS scavenger enzymes, such as Superoxide Dismutase (SOD) (Cervantes Gracia et al., 2017). In animal models, it has been demonstrated that VSMCs have attenuated cGMP production in the presence of O_2^- (Wu et al., 2000). O_2^- also increases PDE5 expression in human VSMCs (Muzaffar et al., 2008). Both mechanisms combined lead to reduced bioavailability of cGMP, which in turn can lead to reduced vasodilatory effects in vasculature. Enhanced ROS activity can

also contribute to other detrimental effects on cells from DNA damage, impaired autophagy, mitochondrial damage and dysregulated inflammatory pathways (Taniyama and Griendling, 2003), all of which could lead to cellular dysfunction. Studies have demonstrated enhanced O_2^- production from endothelial cells in the presence of hyperglycaemia, and more so that this occurs within the mitochondria and enhanced effects of NADPH oxidase (Dymkowska et al., 2014, Nishikawa et al., 2000). Higher concentrations of O_2^- are also seen in placentas from mothers with preeclampsia particularly in early-onset disease (Poston et al., 1995, Sikkema et al., 2001). Nitrotyrosine residues, a proxy measure of $ONOO^-$ activity in tissue have also been shown to be increased in placentae from T1DM (Lyall et al., 1998). Taken in combination with the impaired endothelium-independent vasodilation shown in my work, this suggests a common pathway for vascular dysfunction in the GDM placenta. In order to differentiate the mechanisms further, further work exploring the expression of $ONOO^-$, cGMP and PDE5 in placental tissue and cord blood will be of interest.

The findings in this study are interesting, but it is important to acknowledge the weaknesses of this study. Firstly, it includes a small cohort, recruited prospectively as part of a wider trial. As a result, I have been unable to control for method of delivery and treatment options. Reassuringly, there were no differences in vascular reactivity of CPAs according to method of delivery, a finding which is replicated by others (Mills et al., 2007), nor in treatments used to manage their labour. Due to the prospective enrolment of women (n=60), placental collection was significantly limited by external factors, resulting in just over 50% being available for inclusion.

In conclusion, this novel study demonstrates that arteries on the fetal side of placental circulation have reduced vasodilatory capacity in GDM pregnancy. The effects seem largely to be mediated by alterations in cGMP pathways in vascular smooth muscle, and is negatively associated with hyperglycaemia. This may be an important mechanism to better understand placental health and mechanisms of adverse neonatal outcome in pregnancy complicated by diabetes. I would like to hypothesise that hyperglycaemia results in increased oxidative stress within

the fetoplacental unit, and further mechanistic studies into markers of oxidative stress and cGMP pathways in placental tissue are planned.

7 Discussion and Summary

7.1 Summary of main findings

The key aim of this thesis was to investigate the role of vascular function as a potential pathophysiological mechanism for adverse outcomes in hyperglycaemic pregnancy. I have examined outcomes of pregnancy women with diabetes and further examined in a detailed case control study vascular function on women with GDM. Since many placentally-derived illnesses such as pre-eclampsia and stillbirth occur with higher frequency in pregnancies complicated by diabetes, it was hypothesised that placental vascular function would be altered in pregnancies complicated by GDM. It was also hypothesised that maternal systemic endothelial function would be reduced in such pregnancies. Extensive evidence in systemic vascular beds shows altered endothelial function in those with longstanding diabetes. Preeclampsia the pregnancy unique condition associated with widespread endothelial dysfunction is also increased in diabetes, but risk can be significantly reduced by treatment of hyperglycaemia. I firstly set out to quantify the prevalence of adverse outcomes in pregnancy complicated by pregestational diabetes using our national data. Following the results of this study, I then investigated in the same population the key risk factors associated with stillbirth, an important but poorly understood complication that was up to 5-fold higher in diabetes. Simultaneously, I designed, led and implemented a mechanistic cohort study exploring temporal trends in systemic endothelial function in women with GDM compared to control groups, and after delivery examined vascular reactivity of the fetoplacental resistance arteries in the chorionic plate to determine if vascular function (endothelial and vascular smooth muscle function) was altered in the same cohort.

The main findings from my studies are:

1) **Pregnancies complicated by T1DM and T2DM have significantly higher rates of adverse outcomes compared to non-diabetic controls, and this is not improving over time**

   As detailed in chapter 3, epidemiological data from Scottish births between 1998-2013 showed higher rates of operative delivery (2-3 fold), preterm delivery (3-6 fold), infant overgrowth (4-5 fold) and stillbirths (4-5 fold) in pregnancies complicated by T1DM and T2DM compared to pregnancies without diabetes. Mothers with T1DM had the highest rates of operative and preterm delivery and birthed larger infants, whilst mothers with T2DM had the highest rates of stillbirth. In clinical practice, T1DM can be more challenging to optimise glycaemia than in T2DM leading to fetal hyperinsulinaemia and may be an explanation for the highest adverse outcomes seen in our T1DM population. Despite anecdotal suggestion that T2DM is often a more easily controlled state of hyperglycaemia, they had the highest rates of stillbirth suggesting that coexisting vascular and metabolic risk factors associated with the T2DM phenotype may be contributing to risk, in addition to glycaemia.

2) **In T1DM and T2DM, higher levels of glycaemia are seen in pregnancies that end in stillbirth compared to livebirth, and although glycaemia during pregnancy (as measured by HbA1c) is better in T2DM than T1DM, rates of stillbirth are higher in T2DM.**

   Chapter 4 demonstrates that in pregnancies complicated by T1DM and T2DM, the main modifiable risk factors associated with stillbirth were hyperglycaemia (as measured by HbA1c) and higher maternal BMI in T2DM. Importantly, these effects on stillbirth risk were independent of each other. Strongest glycaemic associations were seen in later pregnancy in the case of T1DM although the association was present in all trimesters, whilst in T2DM the glycaemic association was restricted to the

preconception period. This suggests that hyperglycaemia in pregnancy contributes to stillbirth risk. From work by others, we know that in many cases of stillbirth in diabetes, the exact cause of fetal demise is not known. However, a high proportion of women will have subclinical evidence of vascular defects on histopathological examination of placentas in diabetes, regardless of pregnancy outcome. The association with higher glycaemia and stillbirth risk in T2DM especially suggests detrimental effects around the time of implantation which could lead to impaired invasion and placental vascular development. Taken together, higher glycaemia is associated with stillbirth, a complication of placental vascular insufficiency and supports the hypothesis that glycaemia may contribute to altered placental vascular function in these pregnancies.

Other important risk factors identified in this study included birthweights at the extremes of centiles (<10^{th} and >95^{th} centile) and delivery beyond 38 weeks gestation. Low birthweight may be a sign of placental failure, whilst larger and older babies may place more demand on the placental circulation.

3) **Maternal systemic endothelial-dependent vasodilatory function (as measured by FMD brachial artery) is not different in pregnancies complicated by diabetes compared to non-diabetic pregnancies.**

Studies in chapter five demonstrated similar endothelial-dependent vasodilatation in women with diabetes and controls. Similarly, there was no effect of fasting plasma glucose, insulin resistance measurements or HbA1c on endothelial-dependent vasodilatation. Only three women in this study developed preeclampsia and so it would be wrong to make inferences on shared pathology between preeclampsia and GDM, but my data show that hyperglycaemia in pregnancy itself is not associated with clinically meaningful macrovascular systemic endothelial dysfunction that results in altered vasodilatory capacity. This study examined only one aspect of endothelial function. Retrospectively, it would have been

interesting to quantify multiple markers of endothelial function. Biochemical measures of NO metabolism may have been useful to measure although are confounded by NO production from other sources including dietary consumption. There may also be alterations in the non-vasodilatory endothelial pathways such as procoagulant factors and cellular adhesion molecules. Microvascular endothelial function was also not assessed and would be of interest given the strong relationship of hyperglycaemia and microangiopathy.

4) **In GDM pregnancy, higher fasting plasma glucose is strongly associated with higher SBP and DBP suggesting adverse effects of glucose on vascular function that are non-NO mediated.**

In chapter 5, subgroup analysis of the GDM group revealed a strong association with fasting plasma glucose and higher BP. Whilst endothelial-dependent vasorelaxation did not appear to be impaired in these women, this effect suggests that hyperglycaemia is associated with a pro-contractile phenotype but via alternative pathways to endothelial NO release. Research into other vascular pathways including ROS generation and VSMC function may reveal a common vascular pathophysiological mechanism to GDM and complications such as preeclampsia.

5) **Fetoplacental arteries from GDM subjects show diminished cGMP-mediated vasodilation of VSMCs compared with controls. This effect is negatively associated with higher fasting plasma glucose suggesting a pathophysiological mechanism for GDM and complication associated with placental vascular insufficiency such as preeclampsia and stillbirth.**

There is a paucity of data in human studies of vascular reactivity in hyperglycaemic pregnancy (GDM or pregestational DM). Chapter 6 demonstrates that vasodilatory response to endothelial-independent agonist, SNP, is reduced in pregnancies complicated by GDM suggesting a

pro-contractile phenotype of the fetoplacental resistance vessels. Higher resistance in the fetoplacental vasculature is associated with impaired fetoplacental perfusion and may be a mechanism for placentally-derived disease in GDM. These vascular effects seem to be mediated mainly through glucose effects on cGMP pathways. Further work to establish if these are due to altered cGMP expression, breakdown by PDEs, or ROS generation is warranted. It is well recognised in non-pregnant subjects with longstanding diabetes that chronic hyperglycaemia alters these pathways. Temporary hyperglycaemia may have similar effects and contribute to placental disease in GDM.

7.2 Challenges

1) **Limitations of clinical databases**

My work benefited from the data infrastructure within NHS Scotland. The clinical databases used nationally are used universally, validated for key aspects of data capture and are well integrated into pathways that make them accessible for epidemiological research. However, all databases have their limitations. Clinical databases are susceptible to misreporting of information which may be for a variety of reasons such as missing information, different historical diagnostic criteria and miscoding. For many of the variables I was interested in, the data was validated. However, preeclampsia diagnosis, an important outcome that was central to the thesis was not validated in the SMR02. Discussions with senior authors who have previously published using the SMR02 preeclampsia coding and who have extensive knowledge of the database for other obstetric studies, suggest that preeclampsia is underreported in SMR02. In a multinational study, Scotland reported preeclampsia rates 1.5-2.5% in the obstetric population, compared to almost double that in the other Westernised populations (Roberts et al., 2011). Importantly, in this publication Scotland was the only database where preeclampsia coding had not been validated, nor was it possible to identify how preeclampsia

diagnosis had been reached. For this reason, preeclampsia was not included as a reliably reported outcome in these studies but clearly was of interest. The risk factor associations study in chapter 4 therefore focused solely on stillbirth, as a condition significantly associated with vascular pathology in diabetes.

Furthermore, within the SCI diabetes database very few were diagnosed as GDM when one would anticipate that approximately 5% of the obstetric population would be diagnosed. This supports the anecdotal evidence I see in clinical practice that GDM is not routinely entered into SCI Diabetes. These studies therefore excluded women with GDM, despite being the most common metabolic disorder seen in pregnancy and a significant burden on population health.

2) Association or causation

All of my studies, whether epidemiological or mechanistic, have demonstrated that hyperglycaemia is associated with adverse outcome pregnancy or altered vascular function. Whilst my analysis is supportive across all chapters of glycaemia being a critical factor in development of these outcomes, they do not definitively prove that hyperglycaemia either causes or predicts placental dysfunction, preeclampsia or stillbirth. In a human model, this would be challenging since metabolic and cardiovascular pathways are often interlinked, human populations and disease are heterogenous and pregnancy is an adapting physiological state. There are a host of unmeasured factors such as genetics and there will be unknown biological variables that are unaccounted for. In a small study such as this, important confounders need to be accounted for, but to explore multiple confounders in tandem would require much larger studies to power such analysis.

3) Placental myography – unique aspects

The Institute of Cardiovascular and Medical Sciences at the University of Glasgow have a unique and expert laboratory for vascular myography studies. They are well published in myography, and it is a proud feature of the Centre of Excellence for cardiovascular research. However, my study was the first to use placental chorionic plate arteries which are more delicate to handle, less contractile and respond to different agonists compared to systemic vessels. I was lucky that I was able to liaise and visit the specialist placental research centre at the University of Manchester and work with Dr Mark Wareing. Dr Wareing and his team had accumulated a wealth of data over years and already optimised placental artery myography protocols. I was able to use these protocols for my study. However, the practical aspects of myography such as dissection and mounting and maintaining viability in these delicate vessels was a large challenge that required months of ongoing practice before I felt comfortable.

Furthermore, the experiments were of 8+ hours duration and should be performed on fresh vessels to ensure viability. A novel aspect of my study was to demonstrate hyperglycaemic effects on maternal and placental vasculature in the same cohort. The nature of pregnancy, delivery and need for immediate placental collection meant that I had to be always available to go to the lab and be ready for these long experiments. For the 18-month period during which my participants delivered their babies, I received telephone calls to let me know of imminent delivery at all times of day. A significant proportion would deliver overnight. Extreme flexibility in work and sleep pattern was required on my part to fulfil this aspect of the study. This was the most challenging aspect of this study but also the most satisfying personally. For the most part, I achieved this. The only absolute times I knew in advance I could not collect samples where when I was on shift for clinical duties and during the period when I was out of the country presenting my research. All participants were

aware on entry to the study that due to the sporadic nature of delivery, there was a risk that some placental collections may be missed.

I was on target to achieve collection in two thirds of my cohort, but sustained an injury in the final months that significantly restricted my mobility. For a 5-week period, during which six participants delivered, I was not safe to transport samples nor work in the laboratory safely. The final number of placentas included (32 out of 60) was still a good number to include in a myography study but as result, I had to condense some of my analysis into two groups (GDM versus non-GDM).

4) **Prospective recruitment in pregnancy and placental studies**

Pregnancy is a dynamic situation and clinical staff will make clinical decisions throughout the duration of pregnancy. Recruitment strategies were designed to limit effects of concurrent morbidity and medication use at entry of study, but it does not negate clinical decisions taken throughout pregnancy. None of my population were prescribed definitively vasoactive medication (other than Aspirin) throughout the pregnancy period that required FMD measurement, but some were taking iron supplementation, laxatives and antacids to treat common pregnancy complaints at various points. Whilst none of these are expected to alter vascular function, effects have not been formally tested. Temporal trend observational studies in pregnancy are at risk of bias. This was evident in my placental studies. My study was novel in that it investigated maternal and placental vascular function in the same pregnant cohorts. The advantage of this approach was that I could demonstrate vascular dysfunction within the placenta, despite similar vascular function systemically. However, I could not control for various factors across pregnancy such as method of delivery nor medications used during it. Whilst my, and others analysis, have demonstrated that neither method of delivery, nor medications used peri-partum had significant effect on the vascular reactivity results, it does not completely negate any effect. A

cleaner experiment would have involved including recruiting both cohorts separately, and only including women undergoing elective caesarean section without need for peri-partum medications in the placental study.

5) **Recruitment**

There are two tertiary obstetric centres within NHS Greater Glasgow and Clyde, the Princess Royal Maternity covering North-East Glasgow and the Queen Elizabeth University Hospital covering South-West. These hospitals serve regions with different population demographics. For example, the Princess Royal Maternity serves some of the more materially deprived areas in Scotland, whilst the Queen Elizabeth University Hospital serves some regions with higher ethnic diversity. I was keen to recruit from both, but all study visits had to be conducted at the Queen Elizabeth University Hospital. The reasons for this are that the FMD technology was based at the Queen Elizabeth University Hospital Clinical Research Facility. Attempts were made to recruit from both hospitals, but despite the coverage of travel expenses, women who stayed in areas served by the Princess Royal Maternity were more reluctant to enter the study with travel being cited as a common reason. The overall demographic of the study population is mixed, and I do not think recruitment bias impacts on the study results. However, we should be aiming to overcome obstacles that prevent participants from entering studies from a wide variety of backgrounds, particularly if their demographics are key risk factors in the conditions that we are investigating.

7.3 Future work

There are multiple facets of additional studies that I think are needed to progress understanding in pathophysiology and prevention of obstetric complications in diabetes, and mechanisms for altered placental vascular function. I have summarised further work that I think is important to improving outcomes in these pregnancies, or enhance our understanding of vascular

mechanisms of disease in these pregnancies. Some of this I have already initiated as part of my clinical lectureship.

7.3.1 Epidemiological studies

1) **Validation of preeclampsia in national databases**

   It is important for us to be able to quantify the impact that preeclampsia has on public health nationally. However, previous extracts of the linked databases are limited in allowing us to do this, although we have not defined the extent of underreporting in these databases. A study exploring random samples of women (with and without diabetes) from each maternity centre in Scotland and comparing coded diagnosis in SMR02 against clinical records would be useful. From this we could provide an estimate of how many are incorrectly coded as preeclampsia, but also how many who met diagnostic criteria were not coded. This would afford us better estimates of prevalence nationally. This would require a large, multicentre study with unblinding of individual level data so would need to be performed as part of a multi-centre audit with individual clinical teams collecting data.

2) **Cause of stillbirth – national audit**

   This work has demonstrated that higher glycaemia in pregnancy is associated with stillbirth. However, does not define the causes of stillbirth. From audits performed in other nations, we surmise that upwards of 50% will have no clear identifiable cause of stillbirth, but a majority will show evidence of placental vasculopathy. I am currently leading on a national paper audit of stillbirths in diabetes, in collaboration with the individual regional representatives on the Scottish Diabetes Group Pregnancy Subgroup to better define this in the Scottish population. Each regional representative has agreed to review their clinical notes from 2010-2018 of women with diabetes who experienced stillbirth. From this, they will collect information on: pre-pregnancy

preparation, glycaemic control during pregnancy, medication use, microvascular complications, comorbid illness, maternal obstetric complications, suggested cause of stillbirth and importantly available post-mortem and placental pathology results. Data will then be collated in anonymised format and allow us to identify if achieving obstetric care targets in these women, identify specific causes of stillbirth and decide if alterations in care pathways are needed to improve upon this stubbornly high poor outcome.

3) **Reanalysis of outcomes following nationally funded strategies to improve pregnancy outcomes**

The results from chapter 3 and 4 have helped inform the Scottish Diabetes Improvement Plan strategies in 2021-2026. This, alongside the landmark CONCEPTT trial from Prof Helen Murphy, has helped secure £5million in national funding in Scotland, part of which will help to improve access to CGM in pregnancy for all mothers with T1DM. In future years, it would be important to reanalyse our obstetric outcome data for improvement in obstetric outcomes in this group following widespread use of this technology. Mothers with T2DM and other types of diabetes will however not be automatically afforded access to CGM during pregnancy due to lack of studies in these cohorts. For this group, optimisation of pre-pregnancy weight and metabolic health is likely to be key. The Scottish Diabetes Prevention Programme has been developed and includes £40 million funding over 5 years to impede the rising prevalence of diabetes and obesity related complications in Scotland. Improved and equitable access to evidence-based weight management programmes for at risk individuals is a key component of the prevention programme. GDM and T2DM are identified within these cohorts. Currently, access to weight management service has been agreed post-GDM diagnosis and will occur post-pregnancy, so is unlikely to have an immediate effect on pregnancy outcomes, but with time, an improvement in the health of women of reproductive age will hopefully lead to a reduction in adverse outcomes in

such pregnancies. Furthermore, awareness campaigns and empowering the public with the knowledge of their diabetes risk may trigger some to adopt changes in lifestyle and seek support for their weight. Metabolic pre-conceptual clinical services also need to be better developed in many areas to afford women the best chance of entering pregnancy with optimal chances of a good outcome. Reanalysis of obstetric outcomes following implementation of these strategies will be important in future years.

4) **Optimal timing of delivery to prevent stillbirth and neonatal morbidity in pregnancies complicated by diabetes**

My work has shown that stillbirth rates per 1,000 ongoing pregnancies at that particular gestation peak at 38-39 weeks in T1DM and T2DM. Current national guidance suggests that women are counselled for delivery from 38 weeks onwards. The question is therefore raised if stillbirths could be prevented by lowering that recommendation to 37 weeks. However, there are already high numbers of these women experiencing pre-term delivery and the risk of neonatal morbidity at delivery at 37 weeks compared to 38 weeks needs to be quantified. In chapter 4.4, I simplistically estimate that stillbirth rates could be reduced by 6 per 1,000 births (1-2 stillbirths per year) with an estimated additional 12 cases per year of RDS caused as result. At this gestation, many of these are likely to be of milder severity. However, this estimate is based on prevalence rates in the general obstetric population and assumes delivery of prenatal steroids for lung maturation. In diabetes, fetal lung maturation can be delayed and so this may be a gross underestimate. Numerous studies are required before recommendations can be made to lower recommended gestational age at delivery.

In the first instance, an observational study using national neonatal clinical databases (SMR01, SMR02 and BadgerNet™) to quantify neonatal mortality and serious morbidity according to week of delivery in the

population with diabetes would be useful. Identification of a time point where neonatal mortality exceeds severe morbidity rates would help identify optimal timing of delivery in diabetes. The best methodology for determining optimal timing of delivery in this population would be a very large, multi-national trial that randomised women with diabetes to delivery at 37 or 38 weeks in otherwise healthy pregnancies. However, any such trial would be challenging to conduct logistically, would require robust evidence of morbidity at each gestational age, and would be fraught with multiple ethical dilemmas making it less appealing to researchers and participants. As such, most of our evidence in this area is likely to continue to come from observational data.

7.3.2 Vascular mechanistic studies

My research has suggested altered vascular function in the fetoplacental unit in hyperglycaemic pregnancy. Further mechanistic studies would help consolidate these findings, and simultaneously advance knowledge in mechanisms of placental disease in these pregnancies. Some future studies that I think would be important to complete are:

1) **Understanding biochemical pathways leading to altered VSMC function in placentae from hyperglycaemic pregnancy**

   I have demonstrated impaired NO-independent vasodilatation in fetoplacental arteries in GDM, with a negative association between higher maternal glycaemia and vasodilatory capacity. This implies effects on vascular smooth muscle pathways that control vascular tone, specifically relating to cGMP pathways. Based on my findings, it would be important to explore how key components of these cGMP-mediated vasodilatation are different in GDM pregnancy. As discussed in chapter 6.3.6, these effects could result from reduced bioavailability of cGMP either directly due to reduced cGMP levels via enhanced degradation by increased phosphodiesterase activity. From my studies, I have fresh frozen samples of placental tissue and cord blood. I would be interested to explore this

further. To investigate reduced cGMP levels, I would be interested in performing a number of other experiments. Firstly, quantifying cGMP levels via immunoassay in placenta and cord blood to determine if there is any difference in levels between GDM and non-GDM pregnancy. Mechanisms of reduced cGMP availability could result from enhanced oxidative stress, and scavenging of NO by reactive oxide species. Measuring expression of both superoxide (NO scavenger) and peroxynitrite (superoxide+NO) in these samples could determine if NO scavenger activity is increased in these samples, leading to reduced cGMP production. Furthermore, measurement of other highly toxic reactive ROS such as hydrogen peroxide would be useful to determine if ROS being produced is having a deleterious effect on tissue.

Another potential mechanism for altered cGMP function is through enhanced degradation by PDE enzymes. As discussed in chapter 6, diabetes is known to increase PDE and PDE5 is found in abundance in the placenta. PDE5 quantification in placental tissue and cord blood would allow us to establish if upregulation of PDE5 due to hyperglycaemia is a potential mechanism of placental vascular disease.

2) **miRNA expression in placental tissue as key regulators of vascular homeostasis**

miRNAs are small, non-coding RNAs that have important regulatory functions in various aspects of vascular and endothelial homeostasis. miRNAs have been found to be dysregulated in a number of cardiovascular diseases including preeclampsia (Sheikh et al., 2016). As part of my lectureship, I plan to perform miRNA sequencing on placental tissue from my cohort to identify if there is differential expression of these regulatory miRNAs in GDM placentas, and if so, what gene pathways they regulate. This could provide further insight into placental vascular function, and mechanisms of placental vasculopathy in diabetes.

3) Maternal systemic vascular function

The studies of maternal systemic vascular function contained in this thesis focused primarily on endothelial function, which we know is dysregulated in other obstetric conditions of interest such as preeclampsia. There was no demonstrable difference in this. However, my studies did not include any other vascular phenotyping studies and in hindsight, I think this would be important to look at. An example of an interesting experiment to answer whether non-endothelial dependent vascular function was altered in these pregnancies would be to perform wire myography experiments using systemic maternal vessels. This could use arteries contained within subcutaneous fat from samples taken at the time of caesarean section and measuring contractile or vasodilatory responses to incremental increases in endothelial- dependent and independent vasoactive agonists. Other generic, non-invasive measures of vascular function such as measuring vascular stiffness may also have broadened knowledge of how vasculature behaves in these pregnancies.

7.4 Summary

In summary, this thesis has demonstrated that adverse obstetric outcome is common in pregnancies complicated by diabetes, and there appears to be a dynamic relationship between higher levels of maternal glycaemia and these outcomes. Some of these effects are very well understood and occur as a result of fetal overgrowth due to stimulated fetal hyperinsulinaemia. However, other effects such as stillbirth and preeclampsia are less understood and thought to relate to adverse placental function in such pregnancies. Based on the experiments contained within this thesis, fetoplacental vessels have diminished vasodilatory capacity and may contribute to high vascular resistance within the fetoplacental circulation. These effects appear to be related to cGMP mediated vascular smooth muscle function, but exact mechanisms for alterations in these pathways need to be explored further. This may be an important mechanism of disease in these pregnancies.

In the same cohort, maternal vascular endothelial function was assessed and there was no difference in endothelial NO-dependent vasodilation. However, subgroup analysis of the GDM suggested that higher maternal glycaemia was associated with higher BP suggesting that maternal vascular function is altered in these pregnancies. The mechanisms for this are unlikely to be endothelial in origin, and other vascular pathways independent of NO-release should be explored.

Appendices

Appendix 1: Case report form

Case Report Form

Study ID:

Group:	Gestational Diabetes (GDM)	☐
	Risk factors for GDM	☐
	No risk factors for GDM	☐

SCREENING

Screening Date:

Gestational date at screening:

Inclusion Criteria:	Is patient >18 years old?	☐
	Is this a singleton pregnancy?	☐
	For women undergoing oral glucose tolerance test, is this being done prior to 32 weeks gestation?	☐
	Has the woman had a confirmed normal 20-week anomaly scan?	☐
Exclusion criteria:	Diagnosis of diabetes prior to pregnancy (previous gestational diabetes accepted for gestational diabetes and risk factor groups)	☐
	Diagnosis of hypertension prior to pregnancy	☐
	Diagnosis of pre-existing medical conditions that require vasoactive medications?	☐

Is the patient on any medications other than Aspirin, pregnancy vitamins, folic acid or thyroxine (DISCUSS WITH CI) ☐
Details…….

Known placental or obstetric complications this pregnancy ☐

Does the participant lack capacity to give written informed consent ☐

Is the participant unable to understand written English language ☐

Results of oral glucose tolerance test (if applicable):

Fasting 0 hour glucose ……..
2-hour glucose ……..

HbA1c (if available) ……..

Gestational age at GTT ……

Case Report Form

VISIT 1:

Study ID

Date

Written informed consent complete ☐

Participant Age (whole years) ……

Gestational age (weeks + days) …… Parity …….

Past medical history ……

……

Medications: ……..

……..

Smoking status ……

Any new placental/obstetric complications since screening Y/N if Y details ……

Booking height (m) ……. Booking weight (kg) ……

Current weight (m) …….

BP (mmHg) …….

FMD brachial (%) ……. Attach FMD print out

Bloods	Fasting glucose	☐	Insulin	☐
	Fructosamine	☐	HDL	☐
	IL6	☐	IL8	☐
	TNF alpha	☐	sFlt1	☐
	PlGF	☐		

Case report form

VISIT 2:

Study ID

Date

Gestational age (weeks + days) ……

New medical conditions since last study visit Y/N, if Y details

…….

Medications: ……..

Any new placental/obstetric complications since last study visit? Y/N if Y details …….

BP (mmHg) ……

FMD (%) ……. Attach FMD print out

VISIT 3:

Study ID

Date

Gestational age (weeks + days) ……

New medical conditions since last study visit Y/N, if Y details

…….

Medications: ……..

Any new placental/obstetric complications since last study visit? Y/N if Y details …….

BP (mmHg) ……

FMD (%) ……. Attach FMD print out

Case Report Form

VISIT 4:

Study ID

Date

Gestational age (weeks + days) ……

New medical conditions since last study visit Y/N, if Y details

……

Medications: ……..

……..

Any new placental/obstetric complications since last study visit? Y/N if Y details ……

Weight (m) ……..

BP (mmHg) ……

FMD (%) ……. Attach FMD print out

Bloods	Fasting glucose	☐	Insulin	☐
	Fructosamine	☐	HDL	☐
	IL6	☐	IL8	☐
	TNF alpha	☐	sFlt1	☐
	PlGF	☐		

Note: do not measure blood insulin levels if patient prescribed insulin

Case Report Form

DELIVERY DETAILS:

Study ID

Date of delivery

Gestational age at delivery (weeks + days) ……..

Infant sex ……..

Medical induction Y/N

Prostin Y/N

Syntocin Y/N

Syntometrine Y/N

Mode of delivery (select all that apply)

- vaginal (incl instrumental) ☐
- Elective caesarean section ☐
- Emergency caesarean section ☐

Fetal distress documented? Y/N

Hypertension documented antenatally? Y/N

Pre-eclampsia? Y/N

New medications since study visit 4? Y/N, If yes, please list

Placental weight (grammes):

Vital status of infant:

Placental biopsy taken ☐

Cord blood taken ☐

Appendix 2: Biochemical assay properties

Manufacturer reported properties of assays used in these studies

Assay	Analyser	Lower limit of detection	Coefficient of Variation (95% CI)
Glucose (Gluc3) Roche Cobas™	Cobas 311 autoanalyser	0.11 mmol/L	<1.2%
Fructosamine Roche Cobas™	Cobas 311 autoanalyser	14 µmol/L	<2.0%
Triglycerides Roche Cobas™	Cobas 311 autoanalyser	0.1 mmol/L	<2.0%
Cholesterol (Chol2) Roche Cobas™	Cobas 311 autoanalyser	0.1 mmol/L	<1.6%
HDL Cholesterol (HDLC4) Roche Cobas™	Cobas 311 autoanalyser	0.08 mmol/L	<2.2%
Insulin Roche Cobas™	Cobas 311 autoanalyser	0.2 µU/mL	<5%
Albumin (AlbT2) Roche Cobas™	Cobas 311 autoanalyser	2 g/L	<2.2%
HbA1c (A1C3) Roche Cobas™	Cobas 311 autoanalyser	0.18 mmol/L	<1.9%
sFlt-1 (HANG2MAG-12k) Milliplex™	MAGPIX xPONENT 4.2 (Illuminex™)	2.1 pg/mL	<10% intra-assay <15% inter-assay
PlGF (HCVD1MAG-67k) Milliplex™	MAGPIX xPONENT 4.2 (Illuminex™)	9.2 pg/mL	<10% intra-assay <20% inter-assay
TNF-α (HCYTOMAG-60k) Milliplex™	MAGPIX xPONENT 4.2 (Illuminex™)	0.7pg/mL	2.6% intra-assay 13% inter-assay
IL-6 (HCYTOMAG-60k) Milliplex™	MAGPIX xPONENT 4.2 (Illuminex™)	0.9pg/mL	<2% intra assay 18% inter-assay
IL-1β (HCYTOMAG-60k) Milliplex™	MAGPIX xPONENT 4.2 (Illuminex™)	0.8pg/mL	2.3% intra-assay 6.7 inter-assay

Appendix 3: Milliplex™ assay procedures

PlGF assay using Human Cardiovascular Disease Magnetic Bead Panel 1 (HCVD1MAG-67K) assay

Serum samples were prepared as in chapter 2.2.6. Samples were removed from the freezer on day of experiment and allowed to thaw.

ANTIBODY-IMMOBILISED BEAD PREPARATION

1. Antibody-immobilised beads (specific to PlGF) were sonicated for 30 seconds then vortexed for 60 seconds.
2. 150µL of immobilised-bead solution was combined with bead diluent to achieve a total volume of 3mL and then vortexed for 30 seconds.

QUALITY CONTROL SAMPLE PREPARATION

1. Two quality control samples were provided by manufacturer. Low concentration (range 24-51pg/mL) and high concentration (171-355pg/mL). 250µL of deionised water was added to each of these, with vials then inverted, vortexed and allowed to settle for 10 minutes afterwards.

WASH BUFFER PREPARATION

1. Manufacturer provided wash buffer was brought to room temperature before 60mL was diluted with 540mL of deionised water.

SERUM MATRIX PREPARATION

1. 1mL of deionised water was added to manufacturer provided lyophilised serum matrix and allowed to reconstitute completely over 10 minutes, and then mixed by inverting.

STANDARD PREPARATION

1. 250µL of deionised water was added to vial containing manufacturer provided standard solution. It was then mixed by inverting vial and vortexing for 10 seconds. This was standard 7.
2. Serial dilutions were performed with 250µL assay buffer being added to 125µL of the previously prepared standard (e.g. standard 6 was produced by adding 125µL of standard 7 to 250µL buffer), and mixing well. The table below details standard preparation and resultant PlGF concentration.
3. Assay buffer alone was used as standard 0.

Standard number	Volume of diluent	Volume of standard	PlGF concentration (pg/mL)
#7	250µL deionised water	0	2000
#6	250µL assay buffer	125µL standard #7	666.7
#5	250µL assay buffer	125µL standard #6	222.2
#4	250µL assay buffer	125µL standard #5	74.1
#3	250µL assay buffer	125µL standard #4	24.7
#2	250µL assay buffer	125µL standard #3	8.2
#1	250µL assay buffer	125µL standard #2	2.7
#0	250µL assay buffer	0	0

ASSAY PROCEDURE

1. All standards, controls and sample analysis were performed in duplicate.
2. 100µL assay buffer was added to each well of 96-well plate. The plate was sealed and mixed on a plate shaker at room temperature.
3. The plate seal was then removed, and the assay buffer removed by inverting the plate and tapping sharply to remove as much assay buffer as possible.
4. 25µL serum matrix was added to each standard (0-7) and control well.
5. 25µL assay buffer was added to each sample well.
6. 25µL of standard (0-7) and controls was added to the appropriate wells, and 25µL sample to the sample wells.
7. The mixing antibody bead bottle was vortexed for 30 seconds to ensure mixed, and 25µL was added to each well.
8. The plate was then sealed, covered with foil and placed on gentle agitation on a plate shaker for 18 hours overnight in a refrigerated room (4^0C).
9. The well was then placed and secured on a handheld magnetic plate. The plate was allowed to rest for 1 minute to allow the magnetic beads to be attracted firmly to the surface of the well. The plate seal was then removed, and the plate inverted with a light tap to decant the fluid contents of the well. The magnetic antibody beads should remain attached to the well.
10. The plate was then removed from the magnet, wells washed with 200µL of wash buffer, placing on a plate shaker for 30 seconds before being reattached to the magnet and decanting contents as above.
11. This wash step was repeated three times.
12. 50µL detection antibodies (manufacturer provided) were then added to each well before being resealed, covered with foil and agitated on a plate shaker at room temperature for 1 hour.
13. 50µL of Streptavidin-Phycoerythrin (manufacturer provided) into each well before being resealed, covered with foil and agitated on plate shaker at room temperature for 30 minutes.

14. The plate was then removed from the shaker and washed three times as per steps 9-11.
15. 100µL of sheath fluid (manufacturer provided) was added to all wells and the beads resuspended by agitation on plate shaker for 5 minutes.
16. The plates were then run on MAGPIX™ analyser with xPONENT software to analyse 50µL from each well. A Median Fluorescence Intensity was reported from the software and results analysed manually as described in the main methods chapter.

sFlt-1 assay using Human Angiogenesis Magnetic Bead Panel 2 (HANG2MAG-12K) assay

PREPARATION OF SERUM SAMPLES

1. Serum samples were removed from the freezer on day of experiment and allowed to thaw.
2. 20µL serum was combined with 80µL assay buffer to produce a 1 in 5 dilution.

ANTIBODY-IMMOBILISED BEAD PREPARATION

1. Antibody-immobilised beads (specific to PlGF) were sonicated for 30 seconds then vortexed for 60 seconds.
2. 150µL of immobilised-bead solution was combined with bead diluent to achieve a total volume of 3mL and then vortexed for 30 seconds.

QUALITY CONTROL SAMPLE PREPARATION

1. Two quality control samples were provided by manufacturer. Low concentration (range 112-233pg/mL) and high concentration (956-1985pg/mL). 250µL of deionised water was added to each of these, with vials then inverted, vortexed and allowed to settle for 10 minutes afterwards.

WASH BUFFER PREPARATION

1. Manufacturer provided wash buffer was brought to room temperature before 60mL was diluted with 540mL of deionised water.

SERUM MATRIX PREPARATION

1. 1mL of deionised water was added to manufacturer provided lyophilised serum matrix and allowed to reconstitute completely over 10 minutes, and then mixed by inverting.

STANDARD PREPARATION

1. 250µL of deionised water was added to vial containing manufacturer provided standard solution. It was then mixed by inverting vial and vortexing for 10 seconds. This was standard 7.
2. Serial dilutions were performed with 250µL assay buffer being added to 125µL of the previously prepared standard (e.g., standard 6 was produced by adding 125µL of standard 7 to 250µL buffer), and mixing well. The table below details standard preparation and resultant sFlt-1 concentration.
3. Assay buffer alone was used as standard 0.

Standard number	Volume of diluent	Volume of standard	sFlt-1 concentration (pg/mL)
#7	250µL deionised water	0	10000
#6	200µL assay buffer	100µL standard #7	3333
#5	200µL assay buffer	100µL standard #6	1111
#4	200µL assay buffer	100µL standard #5	370
#3	200µL assay buffer	100µL standard #4	123
#2	200µL assay buffer	100µL standard #3	41
#1	200µL assay buffer	100µL standard #2	14
#0	200µL assay buffer	0	0

ASSAY PROCEDURE

1. All standards, controls and sample analysis were performed in duplicate.
2. 200µL assay buffer was added to each well of 96-well plate. The plate was sealed and mixed on a plate shaker at room temperature.
3. The plate seal was then removed, and the assay buffer removed by inverting the plate and tapping sharply to remove as much assay buffer as possible.
4. 25µL of standard (0-7) and controls was added to the appropriate wells, and 25µL of assay buffer to the sample wells.
5. 25µL serum matrix was added to each standard (0-7) and control well.
6. 25µL of the pre-diluted (1:5) sample was added to the sample wells.
7. The mixing antibody bead bottle was vortexed for 30 seconds to ensure mixed, and 25µL was added to each well.
8. The plate was then sealed, covered with foil and placed on gentle agitation on a plate shaker for 18 hours overnight in a refrigerated room (4^0C).
9. The well was then placed and secured on a handheld magnetic plate. The plate was allowed to rest for 1 minute to allow the magnetic beads to be attracted firmly to the surface of the well. The plate seal was then removed, and the plate inverted with a light tap to decant the fluid contents of the well. The magnetic antibody beads should remain attached to the well.
10. The plate was then removed from the magnet, wells washed with 200µL of wash buffer, placing on a plate shaker for 30 seconds before being reattached to the magnet and decanting contents as above.
11. This wash step was repeated three times.
12. 25µL detection antibodies (manufacturer provided) were then added to each well before being resealed, covered with foil and agitated on a plate shaker at room temperature for 1 hour.
13. 25µL of Streptavidin-Phycoerythrin (manufacturer provided) into each well before being resealed, covered with foil and agitated on plate shaker at room temperature for 30 minutes.

14. The plate was then removed from the shaker and washed three times as per steps 10-12.
15. 100µL of sheath fluid (manufacturer provided) was added to all wells and the beads resuspended by agitation on plate shaker for 5 minutes.
16. The plates were then run on MAGPIX™ analyser with xPONENT software to analyse 50µL from each well. A Median Fluorescence Intensity was reported from the software and concentration results analysed manually as described in the main methods chapter. Final concentrations were multiplied by 5 to account for the initial 1:5 dilution.

IL-1β, IL-6 and TNF-α assay using Human Cytokine Magnetic Bead Panel (HCYTOMAG-60K) assay

PREPARATION OF SERUM SAMPLES

1. Serum samples were removed from the freezer on day of experiment and allowed to thaw.

ANTIBODY-IMMOBILISED BEAD PREPARATION

1. Antibody-immobilised beads (specific to PlGF) were sonicated for 30 seconds then vortexed for 60 seconds.
2. 60µL of immobilised-bead solution was combined with bead diluent to achieve a total volume of 3mL and then vortexed for 30 seconds.

QUALITY CONTROL SAMPLE PREPARATION

1. Two quality control samples were provided by manufacturer. Low concentration (range 109-227pg/mL for IL-6, 100-207pg/mL for IL-1β and 110-228pg/mL for TNF-α) and high concentration (518-1076pg/mL for IL-6, 485-1008 pg/mL for IL-1β and 532-1106pg/mL for TNF-α). 250µL of deionised water was added to each of these, with vials then inverted, vortexed and allowed to settle for 10 minutes afterwards.

WASH BUFFER PREPARATION

1. Manufacturer provided wash buffer was brought to room temperature before 60mL was diluted with 540mL of deionised water.

SERUM MATRIX PREPARATION

1. 1mL of deionised water was added to manufacturer provided lyophilised serum matrix and allowed to reconstitute completely over 10 minutes, and then mixed by inverting.

STANDARD PREPARATION

1. 250µL of deionised water was added to vial containing manufacturer provided standard solution. It was then mixed by inverting vial and vortexing for 10 seconds. This was standard 7.
2. Serial dilutions were performed with 250µL assay buffer being added to 125µL of the previously prepared standard (e.g., standard 6 was produced by adding 125µL of standard 7 to 250µL buffer), and mixing well. The table below details standard preparation and resultant sFlt-1 concentration.
3. Assay buffer alone was used as standard 0.

Standard number	Volume of diluent	Volume of standard	Concentration of all analytes (pg/mL)
#6	250µL deionised water	0	10000
#5	200µL assay buffer	50µL standard #6	2000
#4	200µL assay buffer	50µL standard #5	400
#3	200µL assay buffer	50µL standard #4	80
#2	200µL assay buffer	50µL standard #3	16
#1	200µL assay buffer	50µL standard #2	3.2
#0	200µL assay buffer	0	0

ASSAY PROCEDURE

1. All standards, controls and sample analysis were performed in duplicate.
2. 200µL assay buffer was added to each well of 96-well plate. The plate was sealed and mixed on a plate shaker at room temperature.
3. The plate seal was then removed, and the assay buffer removed by inverting the plate and tapping sharply to remove as much assay buffer as possible.
4. 25µL of standard (0-7) and controls was added to the appropriate wells, and 25µL of assay buffer to the sample wells.
5. 25µL serum matrix was added to each standard (0-7) and control well.
6. 25µL of sample was added to the sample wells.
7. The mixing antibody bead bottle was vortexed for 30 seconds to ensure mixed, and 25µL was added to each well.
8. The plate was then sealed, covered with foil and placed on gentle agitation on a plate shaker for 18 hours overnight in a refrigerated room (4^0C).
9. The well was then placed and secured on a handheld magnetic plate. The plate was allowed to rest for 1 minute to allow the magnetic beads to be attracted firmly to the surface of the well. The plate seal was then removed, and the plate inverted with a light tap to decant the fluid contents of the well. The magnetic antibody beads should remain attached to the well.
10. The plate was then removed from the magnet, wells washed with 200µL of wash buffer, placing on a plate shaker for 30 seconds before being reattached to the magnet and decanting contents as above.
11. This wash step was performed twice.
12. 25µL detection antibodies (manufacturer provided) were then added to each well before being resealed, covered with foil and agitated on a plate shaker at room temperature for 1 hour.
13. 25µL of Streptavidin-Phycoerythrin (manufacturer provided) into each well before being resealed, covered with foil and agitated on plate shaker at room temperature for 30 minutes.
14. The plate was then removed from the shaker and washed three times as per steps 9-11.

15. 150µL of sheath fluid (manufacturer provided) was added to all wells and the beads resuspended by agitation on plate shaker for 5 minutes.
16. The plates were then run on MAGPIX™ analyser with xPONENT software to analyse 100µL from each well. A Median Fluorescence Intensity was reported from the software and concentration results analysed manually as described in the main methods chapter.

Appendix 4: Myography experiment solutions

Preparation of Physiological Salt Solution (PSS) and 62.5 mmol potassium-containing physiological salt solution (KPSS)

1. First step is to produce Stock Solution A (regular and high-potassium variants) and Stock Solution B

STOCK SOLUTION A (REGULAR)

1. Fill a 1L beaker two thirds full with deionised water
2. Weigh out 278.2g NaCl and add to the beaker
3. Weigh out 14.0g KCl salt and add to the beaker
4. Weigh out and add 11.84g $MgSO_4.7H_20$ to the beaker
5. Stir beaker on a magnetic stirrer to allow complete dissolution of all salts
6. Decant all of the solution into a 2L volumetric flask and fill to the 2L line with deionised water
7. Gently, invert the capped flask a few times to mix the solution
8. Store in a fridge (4^0C) for maximum 3 months.

HIGH K^+ STOCK SOLUTION A

1. Fill a 1L beaker two thirds full with deionised water
2. Add 91.34g KCl salt to the beaker
3. Weight and add 5.92g $MgSO_4.7H_2O$
4. As above, stir on a magnetic stirrer until all salts dissolved and then decant into 1L volumetric flask
5. Top the flask up to 1L with demonised water before capping and inverting to mix the solution.
6. Store the solution in the fridge (4^0C) for up to 3 months

STOCK SOLUTION B

1. Fill a 2L beaker two thirds full with deionised water
2. Weigh and add 84g of $NaHCO_3$ salt to the beaker
3. Weigh and add 6.4g KH_2PO_4 to the beaker and sir on a magnetic beaker until complete dissolution of all salts
4. Decant the solution into a 2L volumetric flask and fill with demonised water to the 2L volume mark.
5. Cap and gently invert the flask to mix solutions, and then store in a fridge at 4^0C for up to three months.

PREPARATION OF PHYSIOGICAL SALT SOLUTION (PSS)

1. Add 100mL of regular stock solution A to a 2L volumetric flask
2. Measure out 100mL stock solution B and add to the same flask

3. Fill the flask approximately two thirds full with demonised water
4. Weigh out and add 4g of anhydrous glucose to the flask before capping and inverting flask gently to mix the solutions
5. Bubble the mixture gently with 95%O_2/5%CO_2 gas for 10 minutes
6. Add 5mL 1 mmol Calcium Chloride to the flask and bubble again with the above gas for a further 10 minutes.
7. Top up the flask to the 2L mark with demonised water.
8. Cap and invert the flask gently several times to mix the solution
9. Store in the fridge at 4^0C for up to 1 week

PREPARATION OF 62.5 mmol KPSS

1. Add 50mL of the K^+ containing stock solution A to a 1L flask
2. Add 50mL stock solution B to the same flask
3. Add 2g of anhydrous glucose to the flask before capping and gently inverting to mix the solutions
4. Gently bubble the solution with 95%O_2/5%CO_2 for ten minutes
5. Then add 2.5mL 1M Calcium Chloride to the solution before bubbling again with the same gas for 10 minutes.
6. Fill the flask to the 1L mark with deionised water before capping and invert gently to mix.
7. Store in the fridge at 4^0C for up to 1 week.

References

Abalos, E., Duley, L., Steyn, D. W. & Gialdini, C. 2018. Antihypertensive drug therapy for mild to moderate hypertension during pregnancy. *Cochrane Database Syst Rev,* 10**,** CD002252-CD002252.

Aggarwal, P. K., Chandel, N., Jain, V. & Jha, V. 2012. The relationship between circulating endothelin-1, soluble fms-like tyrosine kinase-1 and soluble endoglin in preeclampsia. *J Hum Hypertens,* 26**,** 236-41.

Aghajafari, F., Nagulesapillai, T., Ronksley, P. E., Tough, S. C., O'Beirne, M. & Rabi, D. M. 2013. Association between maternal serum 25-hydroxyvitamin D level and pregnancy and neonatal outcomes: systematic review and meta-analysis of observational studies. *BMJ,* 346**,** f1169.

Ala, Y., Palluy, O., Favero, J., Bonne, C., Modat, G. & Dornand, J. 1992. Hypoxia/reoxygenation stimulates endothelial cells to promote interleukin-1 and interleukin-6 production. Effects of free radical scavengers. *Agents Actions,* 37**,** 134-139.

Alp, N. J. & Channon, K. M. 2004. Regulation of Endothelial Nitric Oxide Synthase by Tetrahydrobiopterin in Vascular Disease. *Arterioscler Thromb Vasc Biol,* 24**,** 413-420.

Amash, A., Holcberg, G., Sapir, O. & Huleihel, M. 2012. Placental secretion of interleukin-1 and interleukin-1 receptor antagonist in preeclampsia: effect of magnesium sulfate. *J Interferon Cytokine Res,* 32**,** 432-41.

American College of Obstetricians and Gynaecologists 2018. ACOG Practice Bulletin No. 190: Gestational Diabetes Mellitus. *Obstet Gynecol,* 131**,** e49-e64.

American College of Obstetricians and Gynaecologists. 2020. Gestational Hypertension and Preeclampsia: ACOG Practice Bulletin, Number 222. *Obstet Gynecol,* 135

American College of Obstetricians and Gynaecologists 2018. ACOG Practice Bulletin No. 201: Pregestational Diabetes Mellitus. *Obstet Gynecol,* 132**,** e228-e248

Amirian, A., Mahani, M. B. & Abdi, F. 2020. Role of interleukin-6 (IL-6) in predicting gestational diabetes mellitus. *Obstet Gynecol Sci,* 63**,** 407-416.

Ang, C., Hillier, C., Cameron, A. D., Greer, I. A. & Lumsden, M. A. 2001. The Effect of Type 1 Diabetes Mellitus on Vascular Responses to Endothelin-1 in Pregnant Women. *J Clin Endocrinol Metab,* 86**,** 4939-4942.

Ang, C., Hillier, C., Johnston, F., Cameron, A., Greer, I. & Lumsden, M. A. 2002. Endothelial function is preserved in pregnant women with well-controlled type 1 diabetes. *BJOG,* 109**,** 699-707.

Angueira, A. R., Ludvik, A. E., Reddy, T. E., Wicksteed, B., Lowe, W. L. & Layden, B. T. 2015. New Insights Into Gestational Glucose Metabolism: Lessons Learned From 21st Century Approaches. *Diabetes,* 64**,** 327.

Anton, L., Merrill, D. C., Neves, L. A. A., Stovall, K., Gallagher, P. E., Diz, D. I., et al. 2008. Activation of Local Chorionic Villi Angiotensin II Levels But Not Angiotensin (1-7) in Preeclampsia. *Hypertension,* 51**,** 1066-1072.
Anwar, H., Fischbacher, C. M., Leese, G. P., Lindsay, R. S., Mcknight, J. A., Wild, S. H. & On Behalf of the Scottish Diabetes Research Network Epidemiology Group. 2011. Assessment of the under-reporting of diabetes in hospital admission data: a study from the Scottish Diabetes Research Network Epidemiology Group. *Diabet Med,* 28**,** 1514-1519.
Aranow, C. 2011. Vitamin D and the immune system. *J Investig Med,* 59**,** 881-886.
Arcaro, G., Cretti, A., Balzano, S., Lechi, A., Muggeo, M., Bonora, E. & Bonadonna, R. C. 2002. Insulin Causes Endothelial Dysfunction in Humans. *Circulation,* 105**,** 576-582.
Atègbo, J. M., Grissa, O., Yessoufou, A., Hichami, A., Dramane, K. L., Moutairou, K., et al. 2006. Modulation of Adipokines and Cytokines in Gestational Diabetes and Macrosomia. *J Clin Endocrinol Metab,* 91**,** 4137-4143.
Aune, D., Saugstad, O. D., Henriksen, T. & Tonstad, S. 2014. Maternal Body Mass Index and the Risk of Fetal Death, Stillbirth, and Infant Death: A Systematic Review and Meta-analysis. *JAMA,* 311**,** 1536-1546.
Aupont, J. E., Akolekar, R., Illian, A., Neonakis, S. & Nicolaides, K. H. 2016. Prediction of stillbirth from placental growth factor at 19–24 weeks. *Ultrasound Obstet Gynecol,* 48**,** 631-635.
Avogaro, A., Albiero, M., Menegazzo, L., De Kreutzenberg, S. & Fadini, G. P. 2011. Endothelial Dysfunction in Diabetes. *Diabetes Care,* 34**,** S285.
Azizieh, F. Y. & Raghupathy, R. G. 2015. Tumor necrosis factor-α and pregnancy complications: a prospective study. *Med princ pract,* 24**,** 165-170.
Baker, P. N., Pipkin, F. B. & Symonds, E. M. 1990. Platelet Angiotensin II Binding and Plasma Renin Concentration, Plasma Renin Substrate and Plasma Angiotensin II in Human Pregnancy. *Clin Sci,* 79**,** 403-408.
Barbagallo, M., Dominguez, L. J., Licata, G., Shan, J., Bing, L., Karpinski, E., et al. 2001. Vascular Effects of Progesterone. *Hypertension,* 37**,** 142-147.
Barret H.L., Nitert M.D., McIntyre H.D., Hague W. M., Callaway L.K.. & Rowan J. 2014. Predictors of Preeclampsia in Women in the Metformin in Gestational Diabetes (MiG) Study. *J Diab Metab,* 5.
Barros, J. S., Bairos, V. A., Baptista, M. G. & Fagulha, J. O. 2001. Immunocytochemical Localization of Endothelin-1 in Human Placenta from Normal and Preeclamptic Pregnancies. *Hypertens Preg,* 20**,** 125-137.
Baz, B., Riveline, J.P. & Gautier, J.F. 2016. Endocrinology of Pregnancy: Gestational diabetes mellitus: definition, aetiological and clinical aspects. *Eur J Endocrinol,* 174**,** R43-R51.
Beck, P. & Daughaday, W. H. 1967. Human Placental Lactogen: Studies of Its Acute Metabolic Effects and Disposition in Normal Man. *J Clin Investig,* 46**,** 103-110.

Berger, A. 2000. Th1 and Th2 responses: what are they? *BMJ,* 321**,** 424.
Berggren, E. K., Boggess, K. A., Stuebe, A. M. & Jonsson Funk, M. 2011. National Diabetes Data Group vs Carpenter-Coustan criteria to diagnose gestational diabetes. *AJOG,* 205**,** 253.e1-253.e7.
Berkane, N., Liere, P., Oudinet, J.-P., Hertig, A., Lefèvre, G., Pluchino, et al. 2017. From Pregnancy to Preeclampsia: A Key Role for Estrogens. *Endoc Rev,* 38**,** 123-144.
Berndt, S., Blacher, S., Perrier D'hauterive, S., Thiry, M., Tsampalas, M., Cruz, A., et al. 2009. Chorionic Gonadotropin Stimulation of Angiogenesis and Pericyte Recruitment. *J Clin Endocrinol Metab,* 94**,** 4567-4574.
Bianchi, C., De Gennaro, G., Romano, M., Aragona, M., Battini, L., Del Prato, S. & Bertolotto, A. 2018. Pre-pregnancy obesity, gestational diabetes or gestational weight gain: Which is the strongest predictor of pregnancy outcomes? *Diabetes Res Clin Pract,* 144**,** 286-293.
Birdir, C., Droste, L., Fox, L., Frank, M., Fryze, J., Enekwe, A., et al. 2018. Predictive value of sFlt-1, PlGF, sFlt-1/PlGF ratio and PAPP-A for late-onset preeclampsia and IUGR between 32 and 37 weeks of pregnancy. *Preg Hypertens,* 12**,** 124-128.
Bodnar, L. M., Catov, J. M., Simhan, H. N., Holick, M. F., Powers, R. W. & Roberts, J. M. 2007. Maternal Vitamin D Deficiency Increases the Risk of Preeclampsia. *J Clin Endocrinol Metab,* 92**,** 3517-3522.
Boeldt, D. S. & Bird, I. M. 2017. Vascular adaptation in pregnancy and endothelial dysfunction in preeclampsia. *J Endocrinol,* 232**,** R27-R44.
Bolego, C., Buccellati, C., Radaelli, T., Cetin, I., Puglisi, L., Folco, G. & Sala, A. 2006. eNOS, COX-2, and prostacyclin production are impaired in endothelial cells from diabetics. *Biochem Biophys Res Comm,* 339**,** 188-190.
Bonellie, S., Chalmers, J., Gray, R., Greer, I., Jarvis, S. & Williams, C. 2008. Centile charts for birthweight for gestational age for Scottish singleton births. *BMC Pregnancy Childbirth,* 8**,** 5-5.
Borst, S. E. 2004. The role of TNF-α in insulin resistance. *Endocrine,* 23**,** 177-182.
Bouvier, D., Forest, J.-C., Dion-Buteau, E., Bernard, N., Bujold, E., Pereira, B. & Giguère, Y. 2019. Association of Maternal Weight and Gestational Weight Gain with Maternal and Neonate Outcomes: A Prospective Cohort Study. *J Clin Med,* 8.
Brain, S. D. & Grant, A. D. 2004. Vascular Actions of Calcitonin Gene-Related Peptide and Adrenomedullin. *Physiol Rev,* 84**,** 903-934.
Brelje, T. C., Scharp, D. W., Lacy, P. E., Ogren, L., Talamantes, F., Robertson, M., et al. 1993. Effect of homologous placental lactogens, prolactins, and growth hormones on islet B-cell division and insulin secretion in rat, mouse, and human islets: implication for placental lactogen regulation of islet function during pregnancy. *Endocrinology,* 132**,** 879-887.

Bromley, C. & Shelton, N. 2010. The Scottish Health Survey: UK Comparisons. The Scottish Government. https://www.gov.scot/publications/scottish-health-survey-topic-report-uk-comparisons/documents/ - last accessed 27/9/2021
Brouillet, S., Hoffmann, P., Chauvet, S., Salomon, A., Chamboredon, S., Sergent, F., et al. 2012. Revisiting the role of hCG: new regulation of the angiogenic factor EG-VEGF and its receptors. *Cell Mol Life Sci,* 69**,** 1537-1550.
Brown, M., Melamed, N., Murray-Davis, B., Hassan, H., Mawjee, K., Barrett, J., et al. 2019. Timing of delivery in women with pre-pregnancy diabetes mellitus: a population-based study. *BMJ Open Diab Res,* 7**,** e000758.
Brown, M. A., Magee, L. A., Kenny, L. C., Karumanchi, S. A., Mccarthy, F. P., Saito, S., et al. 2018. Hypertensive Disorders of Pregnancy. *Hypertension,* 72**,** 24-43.
Brownfoot, F. C., Hastie, R., Hannan, N. J., Cannon, P., Tuohey, L., Parry, L. J., et al. 2016. Metformin as a prevention and treatment for preeclampsia: effects on soluble fms-like tyrosine kinase 1 and soluble endoglin secretion and endothelial dysfunction. *AJOG,* 214**,** 356.e1-356.e15.
Burton, G. J., Fowden, A. L. & Thornburg, K. L. 2016. Placental Origins of Chronic Disease. *Physiol Rev,* 96**,** 1509-1565.
Burton, G. J., Redman, C. W., Roberts, J. M. & Moffett, A. 2019. Pre-eclampsia: pathophysiology and clinical implications. *BMJ,* 366**,** l2381.
Campia, U., Sullivan, G., Bryant, M. B., Waclawiw, M. A., Quon, M. J. & Panza, J. A. 2004. Insulin impairs endothelium-dependent vasodilation independent of insulin sensitivity or lipid profile. *Am J Physiol Heart Circ Physiol,* 286**,** H76-H82.
Caritis, S., Sibai, B., Hauth, J., Lindheimer, M. D., Klebanoff, M., Thom, E., et al. 1998. Low-Dose Aspirin to Prevent Preeclampsia in Women at High Risk. *NEJM,* 338**,** 701-705.
Carpenter, M. W. & Coustan, D. R. 1982. Criteria for screening tests for gestational diabetes. *AJOG*. 144**,** 768-773.
Carty, D. M., Delles, C. & Dominiczak, A. F. 2008. Novel Biomarkers for Predicting Preeclampsia. *Trends Cardiovasc Med,* 18**,** 186-194.
Casson, I. F., Clarke, C. A., Howard, C. V., Mckendrick, O., Pennycook, S., Pharoah, P. O. D., et al. 1997. Outcomes of pregnancy in insulin dependent diabetic women: results of a five year population cohort study. *BMJ,* 315**,** 275.
Celermajer, D. S. 1998. Testing endothelial function using ultrasound. *J Cardiovascular Pharmacol,* 32 Suppl 3**,** S29-32.
Cervantes Gracia, K., Llanas-Cornejo, D. & Husi, H. 2017. CVD and Oxidative Stress. *J Clin Med,* 6.
Cetin, I., Mazzocco, M. I., Giardini, V., Cardellicchio, M., Calabrese, S., Algeri, P., et al. 2017. PlGF in a clinical setting of pregnancies at risk of preeclampsia and/or intrauterine growth restriction. *J Matern Fetal Neonatal Med,* 30**,** 144-149.

Chappell, L. C., Duckworth, S., Seed, P. T., Griffin, M., Myers, J., Mackillop, L., et al. Diagnostic Accuracy of Placental Growth Factor in Women With Suspected Preeclampsia. *Circulation,* 128**,** 2121-2131.
Chau, K., Hennessy, A. & Makris, A. 2017. Placental growth factor and pre-eclampsia. *J Human Hypertens,* 31**,** 782-786.
Chen, D.-B. & Zheng, J. 2014. Regulation of Placental Angiogenesis. *Microcirc,* 21**,** 15-25.
Chiswick, C., Reynolds, R. M., Denison, F., Drake, A. J., Forbes, S., Newby, D. E., et al. 2015. Effect of metformin on maternal and fetal outcomes in obese pregnant women (EMPOWaR): a randomised, double-blind, placebo-controlled trial. *Lancet Diab Endocrinol,* 3**,** 778-786.
Cho, N. H., Shaw, J. E., Karuranga, S., Huang, Y., Da Rocha Fernandes, J. D., Ohlrogge, A. W. & Malanda, B. 2018. IDF Diabetes Atlas: Global estimates of diabetes prevalence for 2017 and projections for 2045. *Diab Res Clin Pract,* 138**,** 271-281.
Choi, J. W., Im, M. W. & Pai, S. H. 2002. Nitric Oxide Production Increases during Normal Pregnancy and Decreases in Preeclampsia. *Ann Clin Lab Sci,* 32**,** 257-263.
Clark, D. E., Smith, S. K., He, Y., Day, K. A., Licence, D. R., Corps, A. N., et al. 1998. A vascular endothelial growth factor antagonist is produced by the human placenta and released into the maternal circulation. *Biol Reprod,* 59**,** 1540-8.
Clausen, T. D., Mathiesen, E., Ekbom, P., Hellmuth, E., Mandrup-Poulsen, T. & Damm, P. 2005. Poor Pregnancy Outcome in Women With Type 2 Diabetes. *Diab Care,* 28**,** 323.
Consigny, P. M. 1991. Vascular Smooth Muscle Contraction and Relaxation: Pathways and Chemical Modulation. *J Vasc Intervent Radiol,* 2**,** 309-317.
Contag, S., Brown, C., Crimmins, S. & Goetzinger, K. 2016. Influence of Birthweight on the Prospective Stillbirth Risk in the Third Trimester: A Cross-Sectional Cohort Study. *AJP Reports,* 06**,** e287-e298.
Cooper, W. O., Hernandez-Diaz, S., Arbogast, P. G., Dudley, J. A., Dyer, S., Gideon, P. S., et al. 2006. Major Congenital Malformations after First-Trimester Exposure to ACE Inhibitors. *NEJM,* 354**,** 2443-2451.
Corretti, M. C., Anderson, T. J., Benjamin, E. J., Celermajer, D., Charbonneau, F., Creager, et al. 2002. Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: A report of the International Brachial Artery Reactivity Task Force. *JACC,* 39**,** 257-265.
Costa, M. A. 2016. The endocrine function of human placenta: an overview. *Reprod BioMed Online,* 32**,** 14-43.
Crowther, C. A., Hiller, J. E., Moss, J. R., Mcphee, A. J., Jeffries, W. S., Robinson, J. S. & Australian Carbohydrate Intolerance Study in Pregnant Women Trial Group. 2005. Effect of treatment of gestational diabetes mellitus on pregnancy outcomes. *NEJM,* 352**,** 2477-2486.

Dani, C. & Corsini, I. 2020. Guidelines for Management of Neonatal Hypoglycemia: Are They Actually Applicable? *JAMA Ped,* 174**,** 638-639.
Darooghegi Mofrad, M., Djafarian, K., Mozaffari, H. & Shab-Bidar, S. 2018. Effect of magnesium supplementation on endothelial function: A systematic review and meta-analysis of randomized controlled trials. *Atherosclerosis,* 273**,** 98-105.
Daskalakis, G., Marinopoulos, S., Krielesi, V., Papapanagiotou, A., Papantoniou, N., Mesogitis, S. & Antsaklis, A. 2008. Placental pathology in women with gestational diabetes. *Acta Obstet Gynecol Scand,* 87**,** 403-407.
De Resende Guimarães, M. F. B., Brandão, A. H. F., De Lima Rezende, C. A., Cabral, A. C. V., Brum, A. P., Leite, H. V. & Capuruço, C. A. B. 2014. Assessment of endothelial function in pregnant women with preeclampsia and gestational diabetes mellitus by flow-mediated dilation of brachial artery. *Arch Gynecol Obstet,* 290**,** 441-447.
De Vivo, A., Baviera, G., Giordano, D., Todarello, G., Corrado, F. & D'anna, R. 2008. Endoglin, PlGF and sFlt-1 as markers for predicting pre-eclampsia. *Acta Obstet Gynecol Scand,* 87**,** 837-42.
De-Regil, L. M., Palacios, C., Lombardo, L. K. & Peña-Rosas, J. P. 2016. Vitamin D supplementation for women during pregnancy. *Cochrane Database Syst Rev***,** Cd008873.
Dearden, L., Bouret, S. G. & Ozanne, S. E. 2018. Sex and gender differences in developmental programming of metabolism. *Mol Metab,* 15**,** 8-19.
Deng, Z., Zhang, L., Tang, Q., Xu, Y., Liu, S. & Li, H. 2020. Circulating levels of IFN-γ, IL-1, IL-17 and IL-22 in pre-eclampsia: A systematic review and meta-analysis. *Eur J Obstet Gynecol Reproduc Biol,* 248**,** 211-221.
Diabetes and Pregnancy Group France. 2003. French Multicentric Survey of Outcome of Pregnancy in Women With Pregestational Diabetes. *Diab Care,* 26**,** 2990.
Di Fulvio, P., Pandolfi, A., Formoso, G., Di Silvestre, S., Di Tomo, P., Giardinelli, A., et al, A. 2014. Features of endothelial dysfunction in umbilical cord vessels of women with gestational diabetes. *Nutr Metab Cardiovasc Dis,* 24**,** 1337-1345.
Dieber-Rotheneder, M., Beganovic, S., Desoye, G., Lang, U. & Cervar-Zivkovic, M. 2012. Complex expression changes of the placental endothelin system in early and late onset preeclampsia, fetal growth restriction and gestational diabetes. *Life Sci,* 91**,** 710-715.
De Valk H.W., van Nieuwaal N.H.G., Visser G.H.A.. 2006. Pregnancy outcome in type 2 diabetes mellitus: a retrospective analysis from the Netherlands. *Rev Diabet Stud,* 3**,** 134-142.
Dobbie, L. J., Mackin, S. T., Hogarth, K., Lonergan, F., Kannenkeril, D., Brooksbank, K. & Delles, C. 2020. Validation of semi-automated flow-mediated dilation measurement in healthy volunteers. *Blood Press Monit,* 25**,** 216-223.
Dong, Y.-L., Chauhan, M., Green, K. E., Vegiraju, S., Wang, H.-Q., Hankins, G. D. V. & Yallampalli, C. 2006. Circulating calcitonin gene-related peptide and its

placental origins in normotensive and preeclamptic pregnancies. *AJOG,* 195, 1657-1667.
Dong, Y.-L., Green, K. E., Vegiragu, S., Hankins, G. D. V., Martin, E., Chauhan, M., et al. 2005. Evidence for Decreased Calcitonin Gene-Related Peptide (CGRP) Receptors and Compromised Responsiveness to CGRP of Fetoplacental Vessels in Preeclamptic Pregnancies. *J Clin Endocrinol Metab,* 90, 2336-2343.
Dong, Y.-L., Vegiraju, S., Chauhan, M., Gangula, P. R. R., Hankins, G. D. V., Goodrum, L. & Yallampalli, C. 2004. Involvement of calcitonin gene-related peptide in control of human fetoplacental vascular tone. *Am J Physiol Heart Circ Physiol,* 286, H230-H239.
Dong, Y. L., Vegiraju, S., Chauhan, M. & Yallampalli, C. 2003. Expression of calcitonin gene-related peptide receptor components, calcitonin receptor-like receptor and receptor activity modifying protein 1, in the rat placenta during pregnancy and their cellular localization. *Mol Hum Reprod,* 9, 481-490.
Dordea, A. C., Sweeney, M., Taggart, J., Lartey, J., Wessel, H., Robson, S. C. & Taggart, M. J. 2013. Differential vasodilation of human placental and myometrial arteries related to myofilament Ca2+-desensitization and the expression of Hsp20 but not MYPT1. *Mol Hum Reprod,* 19, 727-736.
Dorniak-Wall, T., Grivell, R. M., Dekker, G. A., Hague, W. & Dodd, J. M. 2014. The role of L-arginine in the prevention and treatment of pre-eclampsia: a systematic review of randomised trials. *J Hum Hypertens,* 28, 230-235.
Duhig, K. E., Myers, J., Seed, P. T., Sparkes, J., Lowe, J., Hunter, R. M., et al. 2019. Placental growth factor testing to assess women with suspected pre-eclampsia: a multicentre, pragmatic, stepped-wedge cluster-randomised controlled trial. *Lancet,* 393, 1807-1818.
Duley, L. 2009. The Global Impact of Pre-eclampsia and Eclampsia. *Semin Perinatol,* 33, 130-137.
Dunne, F., Brydon, P., Smith, K. & Gee, H. 2003. Pregnancy in women with Type 2 diabetes: 12 years outcome data 1990–2002. *Diabet Med,* 20, 734-738.
Duran, A., Sáenz, S., Torrejón, M. J., Bordiú, E., Del Valle, L., Galindo, M., et al. 2014. Introduction of IADPSG Criteria for the Screening and Diagnosis of Gestational Diabetes Mellitus Results in Improved Pregnancy Outcomes at a Lower Cost in a Large Cohort of Pregnant Women: The St. Carlos Gestational Diabetes Study. *Diab Care,* 37, 2442.
Durković, J. & Mandić, B. 2009. The Importance of Determining Human Placental Lactogen in the Third Trimester of Pregnancy. *J Med Biochem,* 28, 97-100.
Dymkowska, D., Drabarek, B., Podszywałow-Bartnicka, P., Szczepanowska, J. & Zabłocki, K. 2014. Hyperglycaemia modifies energy metabolism and reactive oxygen species formation in endothelial cells in vitro. *Arch Biochem Biophys,* 542, 7-13.
Dørup, I., Skajaa, K. & Sørensen, K. E. 1999. Normal pregnancy is associated with enhanced endothelium-dependent flow-mediated vasodilation. *Am J Physiol Heart Circ Physiol,* 276, H821-H825.

Edwards, A., Springett, A., Padfield, J., Dorling, J., Bugg, G. & Mansell, P. 2013. Differences in post-mortem findings after stillbirth in women with and without diabetes. *Diab Med,* 30**,** 1219-1224.
Ehrenberg, H. M., Mercer, B. M. & Catalano, P. M. 2004. The influence of obesity and diabetes on the prevalence of macrosomia. *Am J Obstet Gynecol,* 191**,** 964-968.
Ekbom, P., Damm, P., Feldt-Rasmussen, B., Feldt-Rasmussen, U., Mølvig, J. & Mathiesen, E. R. 2001. Pregnancy Outcome in Type 1 Diabetic Women With Microalbuminuria. *Diab Care,* 24**,** 1739.
Eriksson, J. G., Kajantie, E., Osmond, C., Thornburg, K. & Barker, D. J. P. 2010. Boys live dangerously in the womb. *Am J Hum Biol,* 22**,** 330-335.
Ernst, L. M. & Faye-Petersen, O. M. 2014. Miscellaneous Placental Pathology (Includes Massive Perivillous Fibrin Deposition, Fetal Thrombotic Vasculopathy, and Placenta Accreta). *In:* Mcmanus, L. M. & Mitchell, R. N. (eds.) *Pathobiology of Human Disease*. San Diego: Academic Press.
Essayan, D. M., Fox, C. C., Levi-Schaffer, F., Alam, R. & Rosenwasser, L. J. 1998. Biologic activities of IL-1 and its role in human disease. *J Allerg Clin Immunol,* 102**,** 344-350.
Euser, A. G. & Cipolla, M. J. 2009. Magnesium Sulfate for the Treatment of Eclampsia. *Stroke,* 40**,** 1169-1175.
Evers, I., De Valk, H., Mol, B., Ter Braak, E. & Visser, G. 2002. Macrosomia despite good glycaemic control in Type I diabetic pregnancy; results of a nationwide study in The Netherlands. *Diabetologia,* 45**,** 1484-1489.
Evers, I. M., De Valk, H. W. & Visser, G. H. A. 2004. Risk of complications of pregnancy in women with type 1 diabetes: nationwide prospective study in the Netherlands. *BMJ,* 328**,** 915.
Evers, I. M., Nikkels, P. G. J., Sikkema, J. M. & Visser, G. H. A. 2003. Placental Pathology in Women with Type 1 Diabetes and in a Control Group with Normal and Large-for-Gestational-Age Infants. *Placenta,* 24**,** 819-825.
Fadl, H. E. & Simmons, D. 2016. Trends in diabetes in pregnancy in Sweden 1998-2012. *BMJ Open Diab Res Care,* 4**,** e000221.
Fahey, E. & Doyle, S. L. 2019. IL-1 Family Cytokine Regulation of Vascular Permeability and Angiogenesis. *Front Immunol,* 10**,** 1426.
Farrar D, Simmonds. M., Griffin S., Duarte A., Lawlor D.A, Sculpher M., et al 2016. The identification and treatment of women with hyperglycaemia in pregnancy: an analysis of individual participant data,systematic reviews, meta-analyses and an economic evaluation. *Health Technol Assess*, 20(86), 1-348.
Feig, D. S., Donovan, L. E., Corcoy, R., Murphy, K. E., Amiel, S. A., Hunt, K. F., , et al. 2017. Continuous glucose monitoring in pregnant women with type 1 diabetes (CONCEPTT): a multicentre international randomised controlled trial. *Lancet,* 390**,** 2347-2359.
Feig, D. S., Hwee, J., Shah, B. R., Booth, G. L., Bierman, A. S. & Lipscombe, L. L. 2014. Trends in incidence of diabetes in pregnancy and serious perinatal

outcomes: a large, population-based study in Ontario, Canada, 1996-2010. *Diab Care,* 37**,** 1590-6.
Feldman, R. K., Tieu, R. S. & Yasumura, L. 2016. Gestational Diabetes Screening: The International Association of the Diabetes and Pregnancy Study Groups Compared With Carpenter-Coustan Screening. *Obstet Gynecol,* 127**,** 10-17.
Ferguson, K. K., Mcelrath, T. F., Chen, Y.-H., Mukherjee, B. & Meeker, J. D. 2014. Longitudinal Profiling of Inflammatory Cytokines and C-reactive Protein during Uncomplicated and Preterm Pregnancy. *Am J Reproduc Immunol,* 72**,** 326-336.
Figueroa, R., Martinez, E., Fayngersh, R. P., Tejani, N., Mohazzab, H. K. & Wolin, M. S. 2000. Alterations in relaxation to lactate and H(2)O(2) in human placental vessels from gestational diabetic pregnancies. *Am J Physiol Heart Circ Physiol,* 278**,** H706-13.
Fitzgerald, D. J., Entman, S. S., Mulloy, K. & Fitzgerald, G. A. 1987. Decreased prostacyclin biosynthesis preceding the clinical manifestation of pregnancy-induced hypertension. *Circulation,* 75**,** 956-963.
Flammer, A. J., Anderson, T., Celermajer, D. S., Creager, M. A., Deanfield, J., Ganz, P., et al. 2012. The Assessment of Endothelial Function. *Circulation,* 126**,** 753-767.
Flenady, V., Koopmans, L., Middleton, P., Frøen, J. F., Smith, G. C., Gibbons, K., et al. 2011. Major risk factors for stillbirth in high-income countries: a systematic review and meta-analysis. *Lancet,* 377**,** 1331-1340.
Foo, F. L., Mceniery, C. M., Lees, C., Khalil, A. on behalf of the International Working Group on Maternal Haemodynamics. 2017. Assessment of arterial function in pregnancy: recommendations of the International Working Group on Maternal Hemodynamics. *Ultrasound Obstet Gynecol,* 50**,** 324-331.
Förstermann, U. & Münzel, T. 2006. Endothelial Nitric Oxide Synthase in Vascular Disease. *Circulation,* 113**,** 1708-1714.
Gardosi, J., Madurasinghe, V., Williams, M., Malik, A. & Francis, A. 2013. Maternal and fetal risk factors for stillbirth: population based study. *BMJ,* 346**,** f108.
Garg, P., Badhwar, S., Jaryal, A. K., Kachhawa, G., Deepak, K. K. & Kriplani, A. 2017. The temporal trend of vascular function in women with gestational diabetes. *Vasc Med,* 22**,** 96-102.
Gariani, K., Egloff, M., Prati, S., Philippe, J., Boulvain, M. & Jornayvaz, F. R. 2019. Consequences of the Adoption of the IADPSG versus Carpenter and Coustan Criteria to Diagnose Gestational Diabetes: A Before-After Comparison. *Exp Clin Endocrinol Diab,* 127**,** 473-476.
Gennari-Moser, C., Khankin, E. V., Escher, G., Burkhard, F., Frey, B. M., Karumanchi, S. et al. 2013. Vascular Endothelial Growth Factor-A and Aldosterone. *Hypertension,* 61**,** 1111-1117.

Gent, J., Bullough, S., Harrold, J., Jackson, R., Woolfall, K., Andronis, L., Kenny, L., et al. 2020. The PLANES study: a protocol for a randomised controlled feasibility study of the placental growth factor (PlGF) blood test-informed care versus standard care alone for women with a small for gestational age fetus at or after 32 + 0 weeks' gestation. *Pilot Feasibility Stud,* 6**,** 179.
George, E. M. & Granger, J. P. 2011. Endothelin: key mediator of hypertension in preeclampsia. *Am J Hypertens,* 24**,** 964-969.
Gewaltig, M. T. & Kojda, G. 2002. Vasoprotection by nitric oxide: mechanisms and therapeutic potential. *Cardiovasc Res,* 55**,** 250-60.
Ghosh, S. K., Raheja, S., Tuli, A., Raghunandan, C. & Agarwal, S. 2013. Can maternal serum placental growth factor estimation in early second trimester predict the occurrence of early onset preeclampsia and/or early onset intrauterine growth restriction? A prospective cohort study. *J Obstet Gynaecol Res,* 39**,** 881-890.
Gibbins, K. J., Silver, R. M., Pinar, H., Reddy, U. M., Parker, C. B., Thorsten, V., Willinger, M., Dudley, D. J., et al. 2016. Stillbirth, hypertensive disorders of pregnancy, and placental pathology. *Placenta,* 43**,** 61-68.
Godfrey, L. M., Erramouspe, J. & Cleveland, K. W. 2012. Teratogenic Risk of Statins in Pregnancy. *Ann Pharmacother,* 46**,** 1419-1424.
Gomolak, J. R. & Didion, S. P. 2014. Angiotensin II-induced endothelial dysfunction is temporally linked with increases in interleukin-6 and vascular macrophage accumulation. *Front Physiol,* 5**,** 396.
Gonzalez, C., Rosas-Hernandez, H., Jurado-Manzano, B., Ramirez-Lee, M. A., Salazar-Garcia, S., et al. 2015. The prolactin family hormones regulate vascular tone through NO and prostacyclin production in isolated rat aortic rings. *Acta pharmacol Sinica,* 36**,** 572-586.
Gonzalez, Y., Herrera, M. T., Soldevila, G., Garcia-Garcia, L., Fabián, G., Pérez-Armendariz, E. M., et al. 2012. High glucose concentrations induce TNF-α production through the down-regulation of CD33 in primary human monocytes. *BMC Immunol,* 13**,** 19.
Goodman, R. P., Killam, A. P., Brash, A. R. & Branch, R. A. 1982. Prostacyclin production during pregnancy: Comparison of production during normal pregnancy and pregnancy complicated by hypertension. *Am J Obstet Gynecol,* 142**,** 817-822.
Goulopoulou, S. 2017. Maternal Vascular Physiology in Preeclampsia. *Hypertension,* 70**,** 1066-1073.
Graham, A. M., Rasmussen, J. M., Rudolph, M. D., Heim, C. M., Gilmore, J. H., Styner, M., et al. 2018. Maternal Systemic Interleukin-6 During Pregnancy Is Associated With Newborn Amygdala Phenotypes and Subsequent Behavior at 2 Years of Age. *Biol Psychiatry,* 83**,** 109-119.
Gray, C., Vickers, M. H., Dyson, R. M., Reynolds, C. M. & Berry, M. J. 2015. Magnesium sulfate has sex-specific, dose-dependent vasodilator effects on preterm placental vessels. *Biol Sex Differ,* 6**,** 22.

Groen, B., Links, T. P., Van Den Berg, P. P., Hellinga, M., Moerman, S., Visser, G. H. A., et al. 2013. Similar Adverse Pregnancy Outcome in Native and Nonnative Dutch Women with Pregestational Type 2 Diabetes: A Multicentre Retrospective Study. *ISRN Obstet Gynecol,* 2013, 361435.
Grundmann, M., Haidar, M., Placzko, S., Niendorf, R., Darashchonak, N., Hubel, C. A. & Von Versen-Höynck, F. 2012. Vitamin D improves the angiogenic properties of endothelial progenitor cells. *Am J Physiol Cell Physiol,* 303, C954-62.
Guerin, A., Nisenbaum, R. & Ray, J. G. 2007. Use of Maternal GHb Concentration to Estimate the Risk of Congenital Anomalies in the Offspring of Women with Prepregnancy Diabetes. *Diab Care,* 30, 1920.
Han, C. S., Herrin, M. A., Pitruzzello, M. C., Mulla, M. J., Werner, E. F., Pettker, C. M., et al. 2015. Glucose and Metformin Modulate Human First Trimester Trophoblast Function: a Model and Potential Therapy for Diabetes-Associated Uteroplacental Insufficiency. *Am J Reproduc Immunol,* 73, 362-371.
Haram, K., Svendsen, E. & Abildgaard, U. 2009. The HELLP syndrome: Clinical issues and management. A Review. *BMC Pregnancy Childbirth,* 9, 8.
Harmon, Q. E., Huang, L., Umbach, D. M., Klungsøyr, K., Engel, S. M., Magnus, P., Skjærven, R., et al. 2015. Risk of Fetal Death With Preeclampsia. *Obstet Gynecol,* 125.
Haworth, J. C., Mcrae, K. N. & Dilling, L. A. 1976. Prognosis of Infants of Diabetic Mothers in Relation to Neonatal Hypoglycaemia. *Dev Med Child Neurol,* 18, 471-479.
Hawthorne, G., Robson, S., Ryall, E. A., Sen, D., Roberts, S. H. & Platt, M. P. W. 1997. Prospective population based survey of outcome of pregnancy in diabetic women: results of the Northern Diabetic Pregnancy Audit, 1994. *BMJ,* 315, 279.
Hayward, C. E., Higgins, L., Cowley, E. J., Greenwood, S. L., Mills, T. A., Sibley, C. P. & Wareing, M. 2013. Chorionic plate arterial function is altered in maternal obesity. *Placenta,* 34, 281-287.
CEMACH Confidential Enquiry into Maternal and Child Health 2005. Pregnancy in Women with Type 1 and Type 2 Diabetes in 2002–03, England,Wales and Northern Ireland.
Heazell, A. E. P., Hayes, D. J. L., Whitworth, M., Takwoingi, Y., Bayliss, S. E. & Davenport, C. 2019. Biochemical tests of placental function versus ultrasound assessment of fetal size for stillbirth and small-for-gestational-age infants. *Cochrane Database Syst Rev.* CD012455
Henderson, C. E. & Divon, M. Y. 1998. Combining human placental lactogen with routine glucose challenge tests. *Prim Care Update Ob Gyns,* 5, 189-190.
Hertig, A., Liere, P., Chabbert-Buffet, N., Fort, J., Pianos, A., Eychenne, B., et al. 2010. Steroid profiling in preeclamptic women: evidence for aromatase deficiency. *AJOG,* 203, 477.e1-477.e9.

Hillier, T. A., Pedula, K. L., Ogasawara, K. K., Vesco, K. K., Oshiro, C. E. S., Lubarsky, S. L. & Van Marter, J. 2021. A Pragmatic, Randomized Clinical Trial of Gestational Diabetes Screening. *NEJM,* 384**,** 895-904.
Hod, M., Kapur, A., Sacks, D. A., Hadar, E., Agarwal, M., Di Renzo, G. C., et al. 2015. The International Federation of Gynecology and Obstetrics (FIGO) Initiative on gestational diabetes mellitus: A pragmatic guide for diagnosis, management, and care. *Int J Gynecol Obstet,* 131**,** S173-S211.
Holman, N., Bell, R., Murphy, H. & Maresh, M. 2014. Women with pre-gestational diabetes have a higher risk of stillbirth at all gestations after 32 weeks. *Diabet Med,* 31**,** 1129-1132.
Holmes, V. A., Young, I. S., Patterson, C. C., Pearson, D. W. M., Walker, J. D., Maresh, M. J. A. & Mccance, D. R. 2011. Optimal Glycemic Control, Pre-eclampsia, and Gestational Hypertension in Women With Type 1 Diabetes in the Diabetes and Pre-eclampsia Intervention Trial. *Diab Care,* 34**,** 1683.
Holtan, S. G., Chen, Y., Kaimal, R., Creedon, D. J., Enninga, E. A. L., Nevala, W. K. & Markovic, S. N. 2015. Growth Modeling of the Maternal Cytokine Milieu throughout Normal Pregnancy: Macrophage-Derived Chemokine Decreases as Inflammation/Counterregulation Increases. *J Immunol Res,* 2015**,** 952571.
Houghton, D. J., Shackleton, P., Obiekwe, B. C. & Chard, T. 1984. Relationship of maternal and fetal levels of human placental lactogen to the weight and sex of the fetus. *Placenta,* 5**,** 455-458.
Hughes, R. C. E., Rowan, J. & Florkowski, C. M. 2016. Is There a Role for HbA1c in Pregnancy? *Curr Diab Rep,* 16**,** 5.
Iliodromiti, S., Mackay, D. F., Smith, G. C. S., Pell, J. P., Sattar, N., Lawlor, D. A. & Nelson, S. M. 2017. Customised and Noncustomised Birth Weight Centiles and Prediction of Stillbirth and Infant Mortality and Morbidity: A Cohort Study of 979,912 Term Singleton Pregnancies in Scotland. *PLOS Med,* 14**,** e1002228.
Immonen, I., Siimes, A., Stenman, U. H., Kärkkäinen, J. & Fyhrquist, F. 1983. Plasma renin substrate and oestrogens in normal pregnancy. *Scand J Clin Lab Invest,* 43**,** 61-5.
Information Services Division Scotland 2010. Data quality assurance: Assessment of maternity data (SMR02) 2008-09. NHS National Services Scotland. https://www.isdscotland.org/data_quality_assurance/DQA-Assessment-of-Maternity-Data-SMR02-2008-to-2009.pdf - last accessed 27/9/2021
Information Services Division Scotland. 2012. GPD Support: Scottish Index of Multiple Deprivation. Available: https://www.isdscotland.org/Products-and-Services/GPD-Support/Deprivation/SIMD/ - last accessed 27/9/2021.
Information Services Division Scotland. Births in Scottish Hospitals. https://www.isdscotland.org/Health-Topics/Maternity-and-Births/Births/ - last accessed 27/9/2021
Institute of Medicine and National Research Council Committee to Reexamine IOM Pregnancy Weight Guidelines. 2009. The National Academies Collection: Reports. *In:* Rasmussen, K. M. & Yaktine, A. L. (eds.) *Weight Gain During*

Pregnancy: Reexamining the Guidelines. Washington (DC): National Academies Press (US)
International Association of Diabetes and Pregnancy Study Groups Consensus. 2010. International Association of Diabetes and Pregnancy Study Groups Recommendations on the Diagnosis and Classification of Hyperglycemia in Pregnancy. *Diab Care*, 33, 676-682.
Ismail, K. I., Hannigan, A., O'donoghue, K. & Cotter, A. 2017. Abnormal placental cord insertion and adverse pregnancy outcomes: a systematic review and meta-analysis. *Syst Rev,* 6, 242.
ISRCTN Registry. 2019. *Does repeat placental growth factor blood sample testing reduce harm from pre-eclampsia to babies?* https://www.isrctn.com/ISRCTN85912420?q=parrot&filters=&sort=&offset=1&totalResults=4&page=1&pageSize=10&searchType=basic-search - last accessed 30th June 2021.
Jensen, D. M., Damm, P., Moelsted-Pedersen, L., Ovesen, P., Westergaard, J. G., Moeller, M. & Beck-Nielsen, H. 2004. Outcomes in Type 1 Diabetic Pregnancies. *Diab Care,* 27, 2819.
Dodd, J.M., McLeod, A., Windrim, R.C. & Kingdom, J. 2013. Antithrombotic therapy for improving maternal or infant health outcomes in women considered at risk of placental dysfunction. *Cochrane Database Syst Rev,* 7, CD006780.
Johnstone, F. D., Kelly, R. W., Haddad, N. G. & Prescott, R. J. 1988. Prostacyclin production in pregnancy induced hypertension and diabetic pregnancy. *J Obstet Gynaecol,* 9, 90-93.
Johnstone, F. D., Lindsay, R. S. & Steel, J. 2006. Type 1 Diabetes and Pregnancy: Trends in Birth Weight Over 40 Years at a Single Clinic. *Obstet Gynecol,* 107.
Jovanovic-Peterson, L., Peterson, C. M., Reed, G. F., Metzger, B. E., Mills, J. L., Knopp, R. H. & Aarons, J. H. 1991. Maternal postprandial glucose levels and infant birth weight: The Diabetes in Early Pregnancy Study. *Am J Obstet Gynecol,* 164, 103-111.
Urschel, K. & Cicha, I. 2015. TNF-α in the cardiovascular system: from physiology to therapy. *International J Interferon, Cytokine Mediator Res*.
Kaaja, R. 2008. Predictors and risk factors of pre-eclampsia. *Minerva Ginecol,* 60, 421-9.
Kalinderis, M., Papanikolaou, A., Kalinderi, K., Ioannidou, E., Giannoulis, C., Karagiannis, V. & Tarlatzis, B. C. 2011. Elevated Serum Levels of Interleukin-6, Interleukin-1ß and Human Chorionic Gonadotropin in Pre-eclampsia. *Am J Reproduc Immunol,* 66, 468-475.
Kalkhoff, R. K. 1982. Metabolic effects of progesterone. *AJOG,* 142, 735-738.
Karagouni, E. E., Chryssikopoulos, A., Mantzavinos, T., Kanakas, N. & Dotsika, E. N. 1998. Interleukin-1ß and interleukin-1α may affect the implantation rate of patients undergoing in vitro fertilization–embryo transfer. *Fertil Steril,* 70, 553-559.

Katra, P., Dereke, J., Nilsson, C. & Hillman, M. 2016. Plasma Levels of the Interleukin-1-Receptor Antagonist Are Lower in Women with Gestational Diabetes Mellitus and Are Particularly Associated with Postpartum Development of Type 2 Diabetes. *PLOS ONE,* 11**,** e0155701.
Kernaghan, D., Penney, G. C. & Pearson, D. W. M. 2006. Pregnancy-related care and outcomes for women with Type 1 diabetes in Scotland. *Clin Gov,* 11**,** 114-127.
Kim, Y. J., Park, H. S., Lee, H. Y., Ha, E. H., Suh, S. H., Oh, S. K. & Yoo, H. S. 2006. Reduced l-arginine Level and Decreased Placental eNOS Activity in Preeclampsia. *Placenta,* 27**,** 438-444.
Kitzmiller, J. L., Gavin, L. A., Gin, G. D., Jovanovic-Peterson, L., Main, E. K. & Zigrang, W. D. 1991. Preconception Care of Diabetes: Glycemic Control Prevents Congenital Anomalies. *JAMA,* 265**,** 731-736.
Kleinrouweler, C. E., Wiegerinck, M. M. J., Ris-Stalpers, C., Bossuyt, P. M. M., Van Der Post, J. A. M., Von Dadelszen, P., et al. for the EBM Connect Collboration. 2012. Accuracy of circulating placental growth factor, vascular endothelial growth factor, soluble fms-like tyrosine kinase 1 and soluble endoglin in the prediction of pre-eclampsia: a systematic review and meta-analysis. *BJOG,* 119**,** 778-787.
Knock, G. A. & Poston, L. 1996. Bradykinin-mediated relaxation of isolated maternal resistance arteries in normal pregnancy and preeclampsia. *AJOG,* 175**,** 1668-1674.
Kong, L., Nilsson, I. A. K., Gissler, M. & Lavebratt, C. 2019. Associations of Maternal Diabetes and Body Mass Index With Offspring Birth Weight and Prematurity. *JAMA Pediatr,* 173**,** 371-378.
Kossenjans, W., Eis, A., Sahay, R., Brockman, D. & Myatt, L. 2000. Role of peroxynitrite in altered fetal-placental vascular reactivity in diabetes or preeclampsia. *Am J Physiol Heart Circ Physiol,* 278**,** H1311-9.
Kowalczyk, A., Kleniewska, P., Kolodziejczyk, M., Skibska, B. & Goraca, A. 2015. The Role of Endothelin-1 and Endothelin Receptor Antagonists in Inflammatory Response and Sepsis. *Arch Immunol Ther Exp,* 63**,** 41-52.
Krock, B. L., Skuli, N. & Simon, M. C. 2011. Hypoxia-Induced Angiogenesis: Good and Evil. *Genes Cancer,* 2**,** 1117-1133.
Kroll, J. & Waltenberger, J. 1998. VEGF-A Induces Expression of eNOS and iNOS in Endothelial Cells via VEGF Receptor-2 (KDR). *Biochem Biophys Res Comm,* 252**,** 743-746.
Kumasawa, K., Ikawa, M., Kidoya, H., Hasuwa, H., Saito-Fujita, T., Morioka, Y., et al. 2011. Pravastatin induces placental growth factor (PGF) and ameliorates preeclampsia in a mouse model. *Proc Natl Acad Sci USA,* 108**,** 1451.
Kunath, J., Günther, J., Rauh, K., Hoffmann, J., Stecher, L., Rosenfeld, E., et al. 2019. Effects of a lifestyle intervention during pregnancy to prevent excessive gestational weight gain in routine care – the cluster-randomised GeliS trial. *BMC Med,* 17**,** 5.

Ladfors, L., Shaat, N., Wiberg, N., Katasarou, A., Berntorp, K. & Kristensen, K. 2017. Fetal overgrowth in women with type 1 and type 2 diabetes mellitus. *PLOS ONE,* 12**,** e0187917.
LaMarca, B., Brewer, J., & Wallace, K. 2011. IL-6-induced pathophysiology during pre-eclampsia: potential therapeutic role for magnesium sulfate? : *Int J Interferon, Cytokine, Mediator Res*.
Landon, M. B., Spong, C. Y., Thom, E., Carpenter, M. W., Ramin, S. M. & Casey, B. 2009. A multicenter, randomized trial of treatment for mild gestational diabetes. *NEJM*, 361**,** 1339-1348.
Larré, A. B., Parisotto, A., Rockenbach, B. F., Pasin, D. M., Capellari, C., Escouto, D. C., et al. 2017. Phosphodiesterases and preeclampsia. *Med Hypotheses,* 108**,** 94-100.
Lau, S. Y., Guild, S.-J., Barrett, C. J., Chen, Q., Mccowan, L., Jordan, V. & Chamley, L. W. 2013. Tumor Necrosis Factor-Alpha, Interleukin-6, and Interleukin-10 Levels are Altered in Preeclampsia: A Systematic Review and Meta-Analysis. *Am J Reproduc Immunol,* 70**,** 412-427.
Lauenborg, J., Mathiesen, E., Ovesen, P., Westergaard, J. G., Ekbom, P., Mølsted-Pedersen, L. & Damm, P. 2003. Audit on Stillbirths in Women With Pregestational Type 1 Diabetes. *Diab Care,* 26**,** 1385.
Law, G. R., Ellison, G. T. H., Secher, A. L., Damm, P., Mathiesen, E. R., Temple, R., et al. 2015. Analysis of Continuous Glucose Monitoring in Pregnant Women With Diabetes: Distinct Temporal Patterns of Glucose Associated With Large-for-Gestational-Age Infants. *Diab Care,* 38**,** 1319.
Learmont, J. G. & Poston, L. 1996. Nitric oxide is involved in flow-induced dilation of isolated human small fetoplacental arteries. *AJOG,* 174**,** 583-588.
Lee, C.-L., Chiu, P. C. N., Hautala, L., Salo, T., Yeung, W. S. B., Stenman, U.-H. & Koistinen, H. 2013. Human chorionic gonadotropin and its free ß-subunit stimulate trophoblast invasion independent of LH/hCG receptor. *Mol Cell Endocrinol,* 375**,** 43-52.
Lefevre, M. L. 2014. Low-dose aspirin use for the prevention of morbidity and mortality from preeclampsia: U.S. Preventive Services Task Force recommendation statement. *Ann Intern Med,* 161**,** 819-826.
Lehrskov, L. L. & Christensen, R. H. 2019. The role of interleukin-6 in glucose homeostasis and lipid metabolism. *Semin Immunopathol,* 41**,** 491-499.
Letchworth, A. T. & Chard, T. 1972. Human placental lactogen levels in pre-eclampsia. *J Obstet Gynaecol Br Commonw,* 79**,** 680-3.
Levine, R. J., Maynard, S. E., Qian, C., Lim, K.-H., England, L. J., Yu, K. F., et al. 2004. Circulating Angiogenic Factors and the Risk of Preeclampsia. *NEJM,* 350**,** 672-683.
Li, W., Zhang, S., Liu, H., Wang, L., Zhang, C., Leng, J., et al. 2014. Different Associations of Diabetes With ß-Cell Dysfunction and Insulin Resistance Among Obese and Nonobese Chinese Women With Prior Gestational Diabetes Mellitus. *Diab Care,* 37(9)**,** 2533-2539.

Lind, M., Hayes, A., Caprnda, M., Petrovic, D., Rodrigo, L., Kruzliak, P. & Zulli, A. 2017. Inducible nitric oxide synthase: Good or bad? *Biomed Pharmacother,* 93**,** 370-375.
Lindberg, B. S. & Nilsson, B. A. 1973. Variations in maternal plasma levels of human placental lactogen (HPL) in normal pregnancy and labour. *BJOG,* 80**,** 619-626.
Lopes Van Balen, V. A., Van Gansewinkel, T. A. G., De Haas, S., Van Kuijk, S. M. J., Van Drongelen, J., Ghossein-Doha, C. & Spaanderman, M. E. A. 2017. Physiological adaptation of endothelial function to pregnancy: systematic review and meta-analysis. *Ultrasound Obstet Gynecol,* 50**,** 697-708.
Ludvigsson, J. F., Neovius, M., Söderling, J., Gudbjörnsdottir, S., Svensson, A.-M., Franzén, S., et al. 2018. Periconception glycaemic control in women with type 1 diabetes and risk of major birth defects: population based cohort study in Sweden. *BMJ,* 362**,** k2638.
Lumbers, E. R. & Pringle, K. G. 2013. Roles of the circulating renin-angiotensin-aldosterone system in human pregnancy. *Am J Physiol Regul Integr Comp Physiol,* 306**,** R91-R101.
Lyall, F., Gibson, J. L., Greer, I. A., Brockman, D. E., Eis, A. L. & Myatt, L. 1998. Increased Nitrotyrosine in the Diabetic Placenta: Evidence for oxidative stress. *Diab Care,* 21**,** 1753.
Lyall, F., Greer, I. A., Young, A. & Myatt, L. 1996. Nitric oxide concentrations are increased in the feto-placental circulation in intrauterine growth restriction. *Placenta,* 17**,** 165-8.
Lyall, F., Young, A. & Greer, I. A. 1995. Nitric oxide concentrations are increased in the fetoplacental circulation in preeclampsia. *AJOG,* 173**,** 714-718.
Lynch, T. A., Westen, E., Li, D., Katzman, P. J., Malshe, A. & Drennan, K. 2020. Stillbirth in women with diabetes: a retrospective analysis of fetal autopsy reports. *J Matern-Fetal Neonatal Med***,** 1-8.
Mabie, W. C., Disessa, T. G., Crocker, L. G., Sibai, B. M. & Arheart, K. L. 1994. A longitudinal study of cardiac output in normal human pregnancy. *AJOG,* 170**,** 849-56.
Macintosh, M. C. M., Fleming, K. M., Bailey, J. A., Doyle, P., Modder, J., Acolet, D., Golightly, S. & Miller, A. 2006. Perinatal mortality and congenital anomalies in babies of women with type 1 or type 2 diabetes in England, Wales, and Northern Ireland: population based study. *BMJ,* 333**,** 177.
Mackin, S. T., Nelson, S. M., Kerssens, J. J., Wood, R., Wild, S., Colhoun, H. M., et al. 2018. Diabetes and pregnancy: national trends over a 15 year period. *Diabetologia,* 61**,** 1081-1088.
Mackin, S. T., Nelson, S. M., Wild, S. H., Colhoun, H. M., Wood, R., Lindsay, R. S. on behalf of the SDRN Epidemiology Group and the Scottish Diabetes Group Pregnancy subgroup. 2019. Factors associated with stillbirth in women with diabetes. *Diabetologia,* 62**,** 1938-1947.

Madazli, R., Benian, A., Gümüştaş, K., Uzun, H., Ocak, V. & Aksu, F. 1999. Lipid peroxidation and antoxidants in preeclampsia. *Eur J Obstet Gynecol Reproduc Biol,* 85, 205-208.
Magee, L. A., Von Dadelszen, P., Rey, E., Ross, S., Asztalos, E., Murphy, K. E., et al. 2015. Less-Tight versus Tight Control of Hypertension in Pregnancy. *NEJM,* 372, 407-417.
Mandalà, M. 2020. Influence of Estrogens on Uterine Vascular Adaptation in Normal and Preeclamptic Pregnancies. *Int J Mol Sci,* 21(7), 2592.
Mandrup-Poulsen, T., Pickersgill, L. & Donath, M. Y. 2010. Blockade of interleukin 1 in type 1 diabetes mellitus. *Nat Rev Endocrinol,* 6, 158-66.
Maresh, M. J. A., Holmes, V. A., Patterson, C. C., Young, I. S., Pearson, D. W. M., Walker, J. D. & Mccance, D. R. 2015. Glycemic Targets in the Second and Third Trimester of Pregnancy for Women With Type 1 Diabetes. *Diab Care,* 38, 34.
Maritim, A. C., Sanders, R. A. & Watkins, J. B., 2003. Diabetes, oxidative stress, and antioxidants: a review. *J Biochem Mol Toxicol,* 17, 24-38.
Mathiesen, E. R. 2016. Pregnancy Outcomes in Women With Diabetes - Lessons Learned From Clinical Research: The 2015 Norbert Freinkel Award Lecture. *Diab Care,* 39, 2111-2117.
Mathiesen, E. R., Ringholm, L. & Damm, P. 2011. Stillbirth in diabetic pregnancies. *Best Pract Res Clin Obstet Gynaecol,* 25, 105-111.
Maynard, S. E., Min, J.-Y., Merchan, J., Lim, K.-H., Li, J., Mondal, S., et al. 2003. Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. *J Clin Invest,* 111, 649-658.
Mäṅnik, J., Vaas, P., Rull, K., Teesalu, P., Rebane, T. & Laan, M. 2010. Differential Expression Profile of Growth Hormone/Chorionic Somatomammotropin Genes in Placenta of Small- and Large-for-Gestational-Age Newborns. *J Clin Endocrinol Metab,* 95, 2433-2442.
Mccance, D. R., Holmes, V. A., Maresh, M. J. A., Patterson, C. C., Walker, J. D., Pearson, D. W. M. & Young, I. S. 2010. Vitamins C and E for prevention of pre-eclampsia in women with type 1 diabetes (DAPIT): a randomised placebo-controlled trial. *Lancet,* 376, 259-266.
Mccarthy, A. L., Woolfson, R. G., Evans, B. J., Davies, D. R., Raju, S. K. & Poston, L. 1994. Functional characteristics of small placental arteries. *AJOG,* 170, 945-951.
Mcintyre, H. D., Sacks, D. A., Barbour, L. A., Feig, D. S., Catalano, P. M., Damm, P. & Mcelduff, A. 2016. Issues With the Diagnosis and Classification of Hyperglycemia in Early Pregnancy. *Diab Care,* 39, 53.
Mckinlay, C. J. D., Alsweiler, J. M., Anstice, N. S., Burakevych, N., Chakraborty, A., Chase, J. G., et al for the Children with Hypoglycemia and Their Later Development Study. 2017. Association of Neonatal Glycemia With Neurodevelopmental Outcomes at 4.5 Years. *JAMA Pediatr,* 171, 972-983.

Mcknight, J. A., Morris, A. D., Cline, D., Peden, N., Fischbacher, C., Wild, S. on behalf of the Scottish Diabetes Survey Monitoring Group. 2008. Implementing a national quality assurance system for diabetes care: the Scottish Diabetes Survey 2001–2006. *Diabet Med,* 25**,** 743-746.
Mcknight, J. A., Wild, S. H., Lamb, M. J. E., Cooper, M. N., Jones, T. W., Davis, E. A., et al. 2015. Glycaemic control of Type 1 diabetes in clinical practice early in the 21st century: an international comparison. *Diab Med,* 32**,** 1036-1050.
Mclaughlin, K., Baczyk, D., Potts, A., Hladunewich, M., Parker, J. D. & Kingdom, J. C. P. 2017. Low molecular Weight Heparin Improves Endothelial Function in Pregnant Women at High Risk of Preeclampsia. *Hypertension,* 69**,** 180-188.
Meek, C. L., Corcoy, R., Asztalos, E., Kusinski, L. C., López, E., Feig, D. S., et al. on behlaf of the CONCEPPT Collaborative Group. 2021. Which growth standards should be used to identify large- and small-for-gestational age infants of mothers with type 1 diabetes? A pre-specified analysis of the CONCEPTT trial. *BMC Pregnancy Childbirth,* 21**,** 96.
Meek, C. L., Lewis, H. B., Patient, C., Murphy, H. R. & Simmons, D. 2015. Diagnosis of gestational diabetes mellitus: falling through the net. *Diabetologia*, 58**,** 2003-2012.
Meher, S. & Duley, L. 2006. Progesterone for preventing pre-eclampsia and its complications. *Cochrane Database Syst Rev*.
Merrill, D. C., Karoly, M., Chen, K., Ferrario, C. M. & Brosnihan, K. B. 2002. Angiotensin-(1–7) in normal and preeclamptic pregnancy. *Endocrine,* 18**,** 239-245.
Merviel, P., Müller, F., Guibourdenche, J., Berkane, N., Gaudet, R., Bréart, G. & Uzan, S. 2001. Correlations between serum assays of human chorionic gonadotrophin (hCG) and human placental lactogen (hPL) and pre-eclampsia or intrauterine growth restriction (IUGR) among nulliparas younger than 38 years. *Eur J Obstet Gynecol Reproduc Biol,* 95**,** 59-67.
Metcalfe, A., Hutcheon, J. A., Sabr, Y., Lyons, J., Burrows, J., Donovan, L. E. & Joseph, K. S. 2020. Timing of delivery in women with diabetes: A population-based study. *Acta Obstet Gynecol Scand,* 99**,** 341-349.
Metzger, B. E., Lowe, L. P., Dyer, A. R., Trimble, E. R., Chaovarindr, U. & Coustan, D. R. 2008. Hyperglycemia and adverse pregnancy outcomes. *NEJM,* 358**,** 1991-2002.
Metzger, B. E., Persson, B., Lowe, L. P., Dyer, A. R., Cruickshank, J. K. & Deerochanawong, C. 2010. Hyperglycemia and adverse pregnancy outcome study: neonatal glycemia. *Paediatrics*, 126**,** e1545-e1552.
Middleton, P., Crowther, C. A. & Simmonds, L. 2016. Different intensities of glycaemic control for pregnant women with pre-existing diabetes. *Cochrane Database Syst Rev*.
Mills, J. L., Dersimonian, R., Raymond, E., Morrow, J. D., Roberts Ii, L. J., Clemens, J. D., et al. 1999. Prostacyclin and Thromboxane Changes Predating

Clinical Onset of Preeclampsia - A Multicenter Prospective Study. *JAMA,* 282, 356-362.
Mills, T. A., Baker, P. N. & Wareing, M. 2007. The Effect of Mode of Delivery on Placental Chorionic Plate Vascular Reactivity. *Hypertens Pregnancy,* 26, 201-210.
Mills, T. A., Wareing, M., Bugg, G. J., Greenwood, S. L. & Baker, P. N. 2005. Chorionic plate artery function and Doppler indices in normal pregnancy and intrauterine growth restriction. *Eur J Clin Invest,* 35, 758-764.
Miranda, J., Triunfo, S., Rodriguez-Lopez, M., Sairanen, M., Kouru, H., Parra-Saavedra, M., et al. 2017. Performance of third-trimester combined screening model for prediction of adverse perinatal outcome. *Ultrasound Obstet Gynecol,* 50, 353-360.
Mitchell, J. A. & Kirkby, N. S. 2019. Eicosanoids, prostacyclin and cyclooxygenase in the cardiovascular system. *Br J Pharmacol,* 176, 1038-1050.
Mondal, D., Galloway, T. S., Bailey, T. C. & Mathews, F. 2014. Elevated risk of stillbirth in males: systematic review and meta-analysis of more than 30 million births. *BMC Med,* 12, 220.
Montelongo, A., Lasunción, M. A., Pallardo, L. F. & Herrera, E. 1992. Longitudinal Study of Plasma Lipoproteins and Hormones During Pregnancy in Normal and Diabetic Women. *Diabetes,* 41, 1651.
Morales, D. E., Mcgowan, K. A., Grant, D. S., Maheshwari, S., Bhartiya, D., Cid, M. C., et al. 1995. Estrogen Promotes Angiogenic Activity in Human Umbilical Vein Endothelial Cells In Vitro and in a Murine Model. *Circulation,* 91, 755-763.
Morrens, A., Verhaeghe, J., Vanhole, C., Devlieger, R., Mathieu, C. & Benhalima, K. 2016. Risk factors for large-for-gestational age infants in pregnant women with type 1 diabetes. *BMC Pregnancy Childbirth,* 16, 162.
MRC Vitamin Study Research Group 1991. Prevention of neural tube defects: Results of the Medical Research Council Vitamin Study. *Lancet,* 338, 131-137.
Mtali, Y. S., Lyimo, M. A., Luzzatto, L. & Massawe, S. N. 2019. Hypertensive disorders of pregnancy are associated with an inflammatory state: evidence from hematological findings and cytokine levels. *BMC Pregnancy Childbirth,* 19, 237.
Muglu, J., Rather, H., Arroyo-Manzano, D., Bhattacharya, S., Balchin, I., Khalil, A., et al. 2019. Risks of stillbirth and neonatal death with advancing gestation at term: A systematic review and meta-analysis of cohort studies of 15 million pregnancies. *PLOS Med,* 16, e1002838.
Muralimanoharan, S., Maloyan, A. & Myatt, L. 2016. Mitochondrial function and glucose metabolism in the placenta with gestational diabetes mellitus: role of miR-143. *Clin Sci,* 130, 931-41.
Murphy, H. R. 2019. Continuous glucose monitoring targets in type 1 diabetes pregnancy: every 5% time in range matters. *Diabetologia,* 62, 1123-1128.
Murphy, H. R., Bell, R., Cartwright, C., Curnow, P., Maresh, M., Morgan, M., et al. 2017. Improved pregnancy outcomes in women with type 1 and type 2 diabetes but substantial clinic-to-clinic variations: a prospective nationwide study. *Diabetologia,* 60, 1668-1677.

Murphy, H. R., Howgate, C., O'keefe, J., Myers, J., Morgan, M., Coleman, M. A., et al. 2021. Characteristics and outcomes of pregnant women with type 1 or type 2 diabetes: a 5-year national population-based cohort study. *Lancet Diab Endocrinol,* 9**,** 153-164.
Muzaffar, S., Shukla, N., Bond, M., Sala-Newby, G. B., Newby, A. C., Angelini, G. D. & Jeremy, J. Y. 2008. Superoxide from NADPH oxidase upregulates type 5 phosphodiesterase in human vascular smooth muscle cells: inhibition with iloprost and NONOate. *Br J Pharmacol,* 155**,** 847-856.
Waugh, N., Millard, A., Robertson, L., & Royle, P. 2011. Updating the Scottish Needs Assessment Programme Report on type 2 diabetes. https://www.scotphn.net/wp-content/uploads/2015/10/Part-B-Research-Review-for-Scottish-Public-Health-Network.pdf last accessed 27/9/2021
Nafisa, A., Gray, S. G., Cao, Y., Wang, T., Xu, S., Wattoo, F. H., et al. 2018. Endothelial function and dysfunction: Impact of metformin. *Pharmacol Therap,* 192**,** 150-162.
Nakabayashi, M., Sakura, M., Takeda, Y. & Sato, K. 1998. Elevated IL-6 in midtrimester amniotic fluid is involved with the onset of preeclampsia. *Am J Reproduc Immunol,* 39**,** 329-334.
Namiki, A., Brogi, E., Kearney, M., Kim, E. A., Wu, T., Couffinhal, T., Varticovski, L. & Isner, J. M. 1995. Hypoxia Induces Vascular Endothelial Growth Factor in Cultured Human Endothelial Cells. *J Biol Chem,* 270**,** 31189-31195.
Napso, T., Yong, H. E. J., Lopez-Tello, J. & Sferruzzi-Perri, A. N. 2018. The Role of Placental Hormones in Mediating Maternal Adaptations to Support Pregnancy and Lactation. *Front Physiol,* 9**,** 1091.
National Institute for Health and Care Excellence. 2015. *Diabetes in Pregnancy. Management of Diabetes and Its Complications from Preconception to the Postnatal Period,* NICE.
National Diabetes Data Group. 1979. Classification and diagnosis of diabetes mellitus and other categories of glucose intolerance. *Diabetes,* 28**,** 1039-1057.
National Pregnancy in Diabetes Audit Report 2013. 2014. NHS Digital
National Pregnancy In Diabetes Audit Report 2018: England, Wales and the Isle of Man. 2019. NHS Digital.
National Institute for Health and Care Excellence. 2010. *Hypertension in Pregnancy: The Management of Hypertensive Disorders during Pregnancy*, NICE.
National Institue for Health and Care Excellence, 2013. Hypertension in Pregnancy (QS35).
National Institute for Health and Care Excellence 2016. PlGF-based testing to help diagnose suspected pre-eclampsia (Triage PlGF test, Elecsys immunoassay sFlt-1/PlGF ratio, DELFIA Xpress PlGF 1-2-3 test, and BRAHMS sFlt-1 Kryptor/BRAHMS PlGF plus Kryptor PE ratio). *NICE DG23.*
National Institute for Health and Care Excellence. 2019. Hypertension in pregnancy: Evidence review for prediction of complications in pre-eclampsia. NICE (NG133)

National Records of Scotland 2013. Census 2011: key results on population, ethnicity, identity, language, religion, health, housing and accommodation in Scotland.
NHS Scotland. *Scotland's Health on the Web: Organisations*. https://www.scot.nhs.uk/organisations/ - last accessed 27/9/2021
National Services Scotland Information and Intelligence. Assessment of SMR02 (Maternity Inpatient and Day Case) Data; Scotland 2017-2018. https://www.isdscotland.org/Products-and-Services/Data-Quality/docs/20191023-Assessment-of-SMR02-Data-Scotland-2017-2018.pdf - last accessed 27/9/2021
Nishikawa, T., Edelstein, D., Du, X. L., Yamagishi, S.-I., Matsumura, T., Kaneda, Y., et al. 2000. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature,* 404**,** 787-790.
Ong, S., Lash, G. & Baker, P. N. 2000. Angiogenesis and placental growth in normal and compromised pregnancies. *Best Pract Res Clin Obstet Gynaecol,* 14**,** 969-980.
Ong, S. S., Crocker, I. P., Warren, A. Y. & Baker, P. N. 2002. Functional characteristics of chorionic plate placental arteries from normal pregnant women and women with pre-eclampsia. *Hypertens Pregnancy,* 21**,** 175-183.
Owens, L. A., Sedar, J., Carmody, L. & Dunne, F. 2015. Comparing type 1 and type 2 diabetes in pregnancy- similar conditions or is a separate approach required? *BMC Pregnancy Childbirth,* 15**,** 69.
Owusu Darkwa, E., Djagbletey, R., Sottie, D., Owoo, C., Vanderpuye, N. M., Essuman, R. & Aryee, G. 2018. Serum nitric oxide levels in healthy pregnant women: a case- control study in a tertiary facility in Ghana. *Matern health neonatol perinatol,* 4**,** 3-3.
O'sullivan, J. B. & Mahan, C. M. 1964. Criteria for the oral glucose tolerance test in pregnancy. *Diabetes,* 13**,** 278-285.
Palm, M., Basu, S., Larsson, A., Wernroth, L., Åkerud, H. & Axelsson, O. V. E. 2011. A longitudinal study of plasma levels of soluble fms-like tyrosine kinase 1 (sFlt1), placental growth factor (PlGF), sFlt1: PlGF ratio and vascular endothelial growth factor (VEGF-A) in normal pregnancy. *Acta Obstetr Gynecol Scand,* 90**,** 1244-1251.
Pandey, A. K., Singhi, E. K., Arroyo, J. P., Ikizler, T. A., Gould, E. R., et al. 2018. Mechanisms of VEGF (Vascular Endothelial Growth Factor) Inhibitor–Associated Hypertension and Vascular Disease. *Hypertension,* 71**,** e1-e8.
Pang, Y., Dong, J. & Thomas, P. 2015. Progesterone increases nitric oxide synthesis in human vascular endothelial cells through activation of membrane progesterone receptor-α. *Am J Physiol Endocrinol Metab,* 308**,** E899-E911.
Parast, V. M. & Paknahad, Z. 2017. Antioxidant Status and Risk of Gestational Diabetes Mellitus: a Case-Control Study. *Clin Nutr Res,* 6**,** 81-88.
Parida, S. K., Schneider, D. B., Stoss, T. D., Pauly, T. H. & Mcgillis, J. P. 1998. Elevated Circulating Calcitonin Gene-Related Peptide in Umbilical Cord and

Infant Blood Associated with Maternal and Neonatal Sepsis and Shock. *Pediatr Res,* 43**,** 276-282.
Patel, S., Rauf, A., Khan, H. & Abu-Izneid, T. 2017. Renin-angiotensin-aldosterone (RAAS): The ubiquitous system for homeostasis and pathologies. *Biomed Pharmacother,* 94**,** 317-325.
Pedersen, J. 1952. Diabetes and Pregnancy. Blood Sugar of Newborn Infants. A thesis. Danish Science Press, Copenhagen.
Pedersen, J. O. R. & Brandstrup, E. 1956. Foetal mortality in pregnant diabetics: stricct control of diabetes with conservative obstetric management. *Lancet,* 267**,** 607-610.
Penney, G. C., Mair, G., Pearson, D. W. M. on behalf of the Scottish Diabetes in Pregnancy Group. 2003. Outcomes of pregnancies in women with type 1 diabetes in Scotland: a national population-based study. *BJOG,* 110**,** 315-318.
Persson, M., Norman, M. & Hanson, U. 2009. Obstetric and perinatal outcomes in type 1 diabetic pregnancies: A large, population-based study. *Diab Care,* 32**,** 2005-9.
Persson, M., Pasupathy, D., Hanson, U. & Norman, M. 2011. Birth Size Distribution in 3,705 Infants Born to Mothers With Type 1 Diabetes. *Diab Care,* 34**,** 1145.
Persson, M., Pasupathy, D., Hanson, U. & Norman, M. 2012. Disproportionate body composition and perinatal outcome in large-for-gestational-age infants to mothers with type 1 diabetes. *BJOG,* 119**,** 565-572.
Pettitt, D. J., Knowler, W. C., Baird, H. R. & Bennett, P. H. 1980. Gestational diabetes: infant and maternal complications of pregnancy in relation to third-trimester glucose tolerance in the Pima Indians. *Diab Care,* 3**,** 458-464.
Pinheiro Da Costa, B. E., Scocco, C., Poli De Figueiredo, C. E. & Guimarães, J. A. 2006. Maternal medicine: Increased serum phosphodiesterase activity in women with pre-eclampsia. *BJOG,* 113**,** 577-579.
Poston, L., Mccarthy, A. L. & Ritter, J. M. 1995. Control of vascular resistance in the maternal and feto-placental arterial beds. *Pharmacol Therap,* 65**,** 215-239.
Powe, C. E., Huston Presley, L. P., Locascio, J. J. & Catalano, P. M. 2019. Augmented insulin secretory response in early pregnancy. *Diabetologia,* 62**,** 1445-1452.
Pringle, K. G., Tadros, M. A., Callister, R. J. & Lumbers, E. R. 2011. The expression and localization of the human placental prorenin/renin-angiotensin system throughout pregnancy: Roles in trophoblast invasion and angiogenesis? *Placenta,* 32**,** 956-962.
Prins, J. R., Gomez-Lopez, N. & Robertson, S. A. 2012. Interleukin-6 in pregnancy and gestational disorders. *J Reprod Immunol,* 95**,** 1-14.
Public Health England. Health matters: reproductive health and pregnancy planning. https://www.gov.uk/government/publications/health-matters-reproductive-health-and-pregnancy-planning/health-matters-reproductive-health-and-pregnancy-planning - last accessed 27/9/2021

Qi, X., Gong, B., Yu, J., Shen, L., Jin, W., Wu, Z., et al. 2017. Decreased cord blood estradiol levels in related to mothers with gestational diabetes. *Medicine,* 96.
Quinton, A. E., Cook, C.-M. & Peek, M. J. 2007. A Longitudinal Study Using Ultrasound to Assess Flow-Mediated Dilatation in Normal Human Pregnancy. *Hypertens Pregnancy,* 26**,** 273-281.
Rackham, O., Paize, F. & Weindling, A. M. 2009. Cause of Death in Infants of Women with Pregestational Diabetes Mellitus and the Relationship with Glycemic Control. *Postgrad Med,* 121**,** 26-32.
Raghupathy, R., Al Mutawa, E., Makhseed, M., Azizieh, F. & Szekeres-Bartho, J. 2005. Modulation of cytokine production by dydrogesterone in lymphocytes from women with recurrent miscarriage. *BJOG,* 112**,** 1096-101.
Ramsay, J. E., Simms, R. J., Ferrell, W. R., Crawford, L., Greer, I. A., Lumsden, M.-A. & Sattar, N. 2003. Enhancement of Endothelial Function by Pregnancy. *Diab Care,* 26**,** 475.
Rana, S., Powe, C. E., Salahuddin, S., Verlohren, S., Perschel, F. H., Levine, R. J., et al. 2012. Angiogenic Factors and the Risk of Adverse Outcomes in Women With Suspected Preeclampsia. *Circulation,* 125**,** 911-919.
Ranieri, G., Marech, I., Niccoli Asabella, A., Di Palo, A., Porcelli, M., Lavelli, V., et al. 2017. Tyrosine-Kinase Inhibitors Therapies with Mainly Anti-Angiogenic Activity in Advanced Renal Cell Carcinoma: Value of PET/CT in Response Evaluation. *Int J Mol Sci,* 18.
Ray, J. G., Vermeulen, M. J., Shapiro, J. L. & Kenshole, A. B. 2001. Maternal and neonatal outcomes in pregestational and gestational diabetes mellitus, and the influence of maternal obesity and weight gain: the DEPOSIT* study. *QJM,* 94**,** 347-356.
Raymond, D. & Peterson, E. 2011. A critical review of early-onset and late-onset preeclampsia. *Obstet Gynecol Surv,* 66**,** 497-506.
Registration of Births, Deaths and Marriages (Scotland) Act. Sect. 56. 1965 - https://www.legislation.gov.uk/en/ukpga/1965/49/body/1995-11-01?wrap=true last accessed 27/9/2021
Rebarber, A., Istwan, N. B., Russo-Stieglitz, K., Cleary-Goldman, J., Rhea, D. J., Stanziano, G. J. & Saltzman, D. H. 2007. Increased incidence of gestational diabetes in women receiving prophylactic 17alpha-hydroxyprogesterone caproate for prevention of recurrent preterm delivery. *Diab Care,* 30**,** 2277-80.
Roberge, S., Nicolaides, K., Demers, S., Hyett, J., Chaillet, N. & Bujold, E. 2017. The role of aspirin dose on the prevention of preeclampsia and fetal growth restriction: systematic review and meta-analysis. *Am J Obstet Gynecol,* 216**,** 110-120.e6.
Robert, M. F., Neff, R. K., Hubbell, J. P., Taeusch, H. W. & Avery, M. E. 1976. Association between Maternal Diabetes and the Respiratory-Distress Syndrome in the Newborn. *NEJM* 294**,** 357-360.

Roberts, C. L., Ford, J. B., Algert, C. S., Antonsen, S., Chalmers, J., Cnattingius, S., et al. 2011. Population-based trends in pregnancy hypertension and pre-eclampsia: an international comparative study. *BMJ Open,* 1**,** e000101.
Roberts, J. M. & Redman, C. W. G. 1993. Pre-eclampsia: more than pregnancy-induced hypertension. *Lancet,* 341**,** 1447-1451.
Robinson, C. J., Johnson, D. D., Chang, E. Y., Armstrong, D. M. & Wang, W. 2006. Evaluation of placenta growth factor and soluble Fms-like tyrosine kinase 1 receptor levels in mild and severe preeclampsia. *AJOG,* 195**,** 255-259.
Roland, J. M., Murphy, H. R., Ball, V., Northcote-Wright, J. & Temple, R. C. 2005. The pregnancies of women with Type 2 diabetes: poor outcomes but opportunities for improvement. *Diabet Med,* 22**,** 1774-1777.
Rolnik, D. L., Wright, D., Poon, L. C., O'gorman, N., Syngelaki, A., De Paco Matallana, C., et al. 2017. Aspirin versus Placebo in Pregnancies at High Risk for Preterm Preeclampsia. *NEJM,* 377**,** 613-622.
Rosenstein, M. G., Cheng, Y. W., Snowden, J. M., Nicholson, J. M., Doss, A. E. & Caughey, A. B. 2012. The risk of stillbirth and infant death stratified by gestational age in women with gestational diabetes. *AJOG,* 206**,** 309.e1-309.e7.
Royal College of Obstetrics and Gynaecology 2014. Vitamin D in Pregnancy - Scientific Impact Paper No. 43. *RCOG*.
Rumbold, A., Duley, L., Crowther, C. A. & Haslam, R. R. 2008. Antioxidants for preventing pre-eclampsia. *Cochrane Database Syst Rev,* 2008**,** CD004227-CD004227.
Rusterholz, C., Hahn, S. & Holzgreve, W. 2007. Role of placentally produced inflammatory and regulatory cytokines in pregnancy and the etiology of preeclampsia. *Semin Immunopathol,* 29**,** 151-162.
Saarelainen, H., Kärkkäinen, H., Valtonen, P., Punnonen, K., Laitinen, T., Heiskanen, N., et al. 2012. Flow-mediated vasodilation is not attenuated in hypertensive pregnancies despite biochemical signs of inflammation. *ISRN Obstet Gynecol,* 2012**,** 709464.
Saffer, C., Olson, G., Boggess, K. A., Beyerlein, R., Eubank, C. & Sibai, B. M. 2013. Determination of placental growth factor (PlGF) levels in healthy pregnant women without signs or symptoms of preeclampsia. *Pregnancy Hypertens,* 3**,** 124-132.
Salles, A. M. R., Galvao, T. F., Silva, M. T., Motta, L. C. D. & Pereira, M. G. 2012. Antioxidants for preventing preeclampsia: a systematic review. *Sci World J,* 2012**,** 243476-243476.
Sanghavi, M. & Rutherford, J. D. 2014. Cardiovascular Physiology of Pregnancy. *Circulation,* 130**,** 1003-1008.
Savvidou, M. D., Hingorani, A. D., Tsikas, D., Frölich, J. C., Vallance, P. & Nicolaides, K. H. 2003. Endothelial dysfunction and raised plasma concentrations of asymmetric dimethylarginine in pregnant women who subsequently develop pre-eclampsia. *Lancet,* 361**,** 1511-1517.

Scarborough, P., Morgan, R. D., Webster, P. & Rayner, M. 2011. Differences in coronary heart disease, stroke and cancer mortality rates between England, Wales, Scotland and Northern Ireland: the role of diet and nutrition. *BMJ Open,* 1, e000263.
Schaefer, T. M., Wright, J. A., Pioli, P. A. & Wira, C. R. 2005. IL-1beta-mediated proinflammatory responses are inhibited by estradiol via down-regulation of IL-1 receptor type I in uterine epithelial cells. *J Immunol,* 175, 6509-16.
Schiessl, B., Mylonas, I., Hantschmann, P., Kuhn, C., Schulze, S., Kunze, S., et al. 2005. Expression of endothelial NO synthase, inducible NO synthase, and estrogen receptors alpha and beta in placental tissue of normal, preeclamptic, and intrauterine growth-restricted pregnancies. *J Histochem Cytochem,* 53, 1441-1449.
Schoofs, K., Grittner, U., Engels, T., Pape, J., Denk, B., Henrich, W. & Verlohren, S. 2014. The importance of repeated measurements of the sFlt-1/PlGF ratio for the prediction of preeclampsia and intrauterine growth restriction. *J Perinat Med,* 42, 61-8.
Schulze, F., Wehner, J., Kratschmar, D. V., Makshana, V., Meier, D. T., Häuselmann, S. P., et al. 2020. Inhibition of IL-1beta improves Glycaemia in a Mouse Model for Gestational Diabetes. *Sci Rep,* 10, 3035.
Scottish Intercollegiate Guidelines Network. 2017. SIGN 116: Management of Diabetes.
Scott, E. M., Feig, D. S., Murphy, H. R. & Law, G. R. 2020. Continuous Glucose Monitoring in Pregnancy: Importance of Analyzing Temporal Profiles to Understand Clinical Outcomes. *Diab Care,* 43, 1178.
Scottish Care Information Diabetes Collaboration. SCI Diabetes: Project Overview. https://www.sci-diabetes.scot.nhs.uk/project-overview/ -last accessed 30th June 2021.
Scottish Diabetes Research Network. http://sdrn.org.uk/about/ - last accessed 4/10/2021
Scottish Diabetes Survey Monitoring Group 2014. Scottish Diabetes Survey 2014. http://www.diabetesinscotland.org.uk/wp-content/uploads/2019/12/Diabetes-in-Scotland-website-Scottish-Diabetes-Survey-2014.pdf - last accessed 27/9/2021
Sena, C. M., Leandro, A., Azul, L., Seiça, R. & Perry, G. 2018. Vascular Oxidative Stress: Impact and Therapeutic Approaches. *Front Physiol,* 9, 1668.
Sermer, M., Naylor, C. D. & Gare, D. J. 1995. Impact of increasing carbohydrate intolerance on maternal-fetal outcomes in 3637 women without gestational diabetes: the Toronto tri-hospital gestational diabetes project. *AJOG,* 173, 146-156.
Shah, R., Harding, J., Brown, J. & Mckinlay, C. 2019. Neonatal Glycaemia and Neurodevelopmental Outcomes: A Systematic Review and Meta-Analysis. *Neonatol,* 115, 116-126.

Sharifipour, F., Abedi, P., Ciahkal, S. F., Jahanfar, S., Mohaghegh, Z. & Zahedian, M. 2020. Serum vitamin E level and gestational diabetes mellitus: a systematic review and meta-analysis. *J Diab Metab Disord,* 19**,** 1787-1795.
Sheikh, A. M., Small, H. Y., Currie, G. & Delles, C. 2016. Systematic Review of Micro-RNA Expression in Pre-Eclampsia Identifies a Number of Common Pathways Associated with the Disease. *PLOS ONE,* 11**,** e0160808.
Shieh, C., Cullen, D. L., Pike, C. & Pressler, S. J. 2018. Intervention strategies for preventing excessive gestational weight gain: systematic review and meta-analysis. *Obes Rev,* 19**,** 1093-1109.
Shimaoka, Y., Hidaka, Y. O. H., Tada, H., Amino, N., Nakamura, T., Murata, Y., et al. 2000. Changes in Cytokine Production During and After Normal Pregnancy. *Am J Reproduc Immunol,* 44**,** 143-147.
Shimosawa, T., Ogihara, T., Matsui, H., Asano, T., Ando, K. & Fujita, T. 2003. Deficiency of Adrenomedullin Induces Insulin Resistance by Increasing Oxidative Stress. *Hypertension,* 41**,** 1080-1085.
Shojaati, K., Causevic, M., Kadereit, B., Dick, B., Imobersteg, J., Schneider, H., et al. 2004. Evidence for compromised aldosterone synthase enzyme activity in preeclampsia. *Kidney Int,* 66**,** 2322-2328.
Sibai, B. M. 2004. Diagnosis, controversies, and management of the syndrome of hemolysis, elevated liver enzymes, and low platelet count. *Obstet Gynecol,* 103**,** 981-91.
Sibai, B. M., Caritis, S. N., Thom, E., Klebanoff, M., Mcnellis, D., Rocco, L., et al. 1993. Prevention of Preeclampsia with Low-Dose Aspirin in Healthy, Nulliparous Pregnant Women. *NEJM,* 329**,** 1213-1218.
Sibai, B. M. & Frangieh, A. 1995. Maternal adaptation to pregnancy. *Curr Opin Obstet Gynecol,* 7**,** 420-6.
Siega-Riz, A. M., Viswanathan, M., Moos, M. K., Deierlein, A., Mumford, S., Knaack, J., et al. 2009. A systematic review of outcomes of maternal weight gain according to the Institute of Medicine recommendations: birthweight, fetal growth, and postpartum weight retention. *Am J Obstet Gynecol,* 201**,** 339.e1-14.
Sikkema, J. M., Van Rijn, B. B., Franx, A., Bruinse, H. W., De Roos, R., Stroes, E. S. G. & Van Faassen, E. E. 2001. Placental Superoxide is Increased in Pre-eclampsia. *Placenta,* 22**,** 304-308.
Simmons, D., Thompson, C. F. & Conroy, C. 2000. Incidence and risk factors for neonatal hypoglycaemia among women with gestational diabetes mellitus in South Auckland. *Diabet Med,* 17**,** 830-4.
Small, M., Cameron, A., Lunan, C. B. & Maccuish, A. C. 1987. Macrosomia in pregnancy complicated by insulin-dependent diabetes mellitus. *Diab Care,* 10**,** 594-9.
Sobrevia, L., Nadal, A., Yudilevich, D. L. & Mann, G. E. 1996. Activation of L-arginine transport (system y+) and nitric oxide synthase by elevated glucose and insulin in human endothelial cells. *J Physiol,* 490**,** 775-781.

Sobrevia, L., Yudilevich, D. L. & Mann, G. E. 1998. Elevated D-glucose induces insulin insensitivity in human umbilical endothelial cells isolated from gestational diabetic pregnancies. *J Physiol,* 506**,** 219-230.
Sobrino, A., Oviedo, P. J., Novella, S., Laguna-Fernandez, A., Bueno, C., García-Pérez, M. A., et al. 2010. Estradiol selectively stimulates endothelial prostacyclin production through estrogen receptor-α. *J mol Endocrinol,* 44**,** 237-46.
Sohlberg, S., Wikström, A. K., Olovsson, M., Lindgren, P., Axelsson, O., Mulic-Lutvica, A., Weis, J. & Wikström, J. 2014. In vivo 31P-MR spectroscopy in normal pregnancy, early and late preeclampsia: A study of placental metabolism. *Placenta,* 35**,** 318-323.
Spiers, A. & Padmanabhan, N. 2005. A Guide to Wire Myography. *In:* Fennell, J. P. & Baker, A. H. (eds.) *Hypertension: Methods and Protocols.* Totowa, NJ: Humana Press.
Srinivasan, G., Pildes, R. S., Cattamanchi, G., Voora, S. & Lilien, L. D. 1986. Plasma glucose values in normal neonates: a new look. *J Pediatr,* 109**,** 114-7.
Stacey, T., Tennant, P. W. G., Mccowan, L. M. E., Mitchell, E. A., Budd, J., Li, M., et al. 2019. Gestational diabetes and the risk of late stillbirth: a case–control study from England, UK. *BJOG,* 126**,** 973-982.
Staun-Ram, E. & Shalev, E. 2005. Human trophoblast function during the implantation process. *Reproduc Biol Endocrinol,* 3**,** 56.
Stenninger, E., Flink, R., Eriksson, B. & Sahlèn, C. 1998. Long-term neurological dysfunction and neonatal hypoglycaemia after diabetic pregnancy. *Arch dis childhood. Fetal neonatal ed,* 79**,** F174-9.
Stogianni, A., Lendahls, L., Landin-Olsson, M. & Thunander, M. 2019. Obstetric and perinatal outcomes in pregnancies complicated by diabetes, and control pregnancies, in Kronoberg, Sweden. *BMC Pregnancy Childbirth,* 19**,** 159.
Storment, J. M., Meyer, M. & Osol, G. 2000. Estrogen augments the vasodilatory effects of vascular endothelial growth factor in the uterine circulation of the rat. *Am J Obstet Gynecol,* 183**,** 449-453.
Stutchfield, P., Whitaker, R. & Russell, I. 2005. Antenatal betamethasone and incidence of neonatal respiratory distress after elective caesarean section: pragmatic randomised trial. *BMJ,* 331**,** 662.
Sudhir, K., Jennings, G. L., Funder, J. W. & Komesaroff, P. A. 1996. Estrogen Enhances Basal Nitric Oxide Release in the Forearm Vasculature in Perimenopausal Women. *Hypertension,* 28**,** 330-334.
Sudo, N., Kamoi, K., Ishibashi, M. & Yamaji, T. 1993. Plasma endothelin-1 and big endothelin-1 levels in women with pre-eclampsia. *Acta Endocrinol (Copenh),* 129**,** 114-20.
Swiderski, S., Celewicz, Z., Miazgowski, T. & Ogonowski, J. 2010. Maternal endothelin-1 and cyclic guanosine monophosphate concentrations in pregnancies complicated by pregravid and gestational diabetes mellitus. *Gynecol Obstet Invest,* 69**,** 46-50.

Syngelaki, A., Nicolaides, K. H., Balani, J., Hyer, S., Akolekar, R., Kotecha, R., et al. 2016. Metformin versus Placebo in Obese Pregnant Women without Diabetes Mellitus. *NEJM,* 374**,** 434-443.
Szarka, A., Rigó, J., Lázár, L., Bekő, G. & Molvarec, A. 2010. Circulating cytokines, chemokines and adhesion molecules in normal pregnancy and preeclampsia determined by multiplex suspension array. *BMC Immunol,* 11**,** 59.
Sánchez-Aranguren, L. C., Prada, C. E., Riaño-Medina, C. E. & Lopez, M. 2014. Endothelial dysfunction and preeclampsia: role of oxidative stress. *Front Physiol,* 5**,** 372.
Sørensen, S., Von Tabouillot, D., Schiøler, V., Greisen, G., Petersen, S. & Larsen, T. 2000. Serial measurements of serum human placental lactogen (hPL) and serial ultrasound examinations in the evaluation of fetal growth. *Early Hum Develop,* 60**,** 25-34.
Tan, X., Li, S., Chang, Y., Fang, C., Liu, H., Zhang, X. & Wang, Y. 2016. Effect of metformin treatment during pregnancy on women with PCOS: a systematic review and meta-analysis. *Clin Invest Med,* 39**,** E120-31.
Tanaka, T., Narazaki, M. & Kishimoto, T. 2014. IL-6 in Inflammation, Immunity, and Disease. *Cold Spring Harbor Perspect Biol,* 6.
Taniyama, Y. & Griendling, K. K. 2003. Reactive Oxygen Species in the Vasculature. *Hypertension,* 42**,** 1075-1081.
Tarrade, A., Lai Kuen, R., Malassiné, A., Tricottet, V., Blain, P., Vidaud, M. & Evain-Brion, D. 2001. Characterization of human villous and extravillous trophoblasts isolated from first trimester placenta. *Lab Invest,* 81**,** 1199-211.
Taylor, R. N., Varma, M., Teng, N. N. & Roberts, J. M. 1990. Women with preeclampsia have higher plasma endothelin levels than women with normal pregnancies. *J Clin Endocrinol Metab,* 71**,** 1675-7.
Temple, R. C., Aldridge, V. J. & Murphy, H. R. 2006. Prepregnancy Care and Pregnancy Outcomes in Women With Type 1 Diabetes. *Diab Care,* 29**,** 1744.
Tennant, P. W. G., Glinianaia, S. V., Bilous, R. W., Rankin, J. & Bell, R. 2014. Pre-existing diabetes, maternal glycated haemoglobin, and the risks of fetal and infant death: a population-based study. *Diabetologia,* 57**,** 285-294.
Tiano, J. P. & Mauvais-Jarvis, F. 2012. Importance of oestrogen receptors to preserve functional ß-cell mass in diabetes. *Nat Rev Endocrinol,* 8**,** 342-51.
Toder, V., Fein, A., Carp, H. & Torchinsky, A. 2003. TNF-α in Pregnancy Loss and Embryo Maldevelopment: A Mediator of Detrimental Stimuli or a Protector of the Fetoplacental Unit? *J Assist Reprod Genet,* 20**,** 73-81.
Tomimatsu, T., Mimura, K., Endo, M., Kumasawa, K. & Kimura, T. 2017. Pathophysiology of preeclampsia: an angiogenic imbalance and long-lasting systemic vascular dysfunction. *Hypertens Res,* 40**,** 305-310.
Toth, P., Li, X., Rao, C. V., Lincoln, S. R., Sanfilippo, J. S., Spinnato, J. A. & Yussman, M. A. 1994. Expression of functional human chorionic gonadotropin/human luteinizing hormone receptor gene in human uterine arteries. *J Clin Endocrinol Metab,* 79**,** 307-315.

Tousoulis, D., Kampoli, A. M., Tentolouris, C., Papageorgiou, N. & Stefanadis, C. 2012. The role of nitric oxide on endothelial function. *Curr Vasc Pharmacol,* 10**,** 4-18.
Tranquilli, A. L., Brown, M. A., Zeeman, G. G., Dekker, G. & Sibai, B. M. 2013. The definition of severe and early-onset preeclampsia. Statements from the International Society for the Study of Hypertension in Pregnancy (ISSHP). *Pregnancy Hypertens,* 3**,** 44-47.
Turco, M. Y. & Moffett, A. 2019. Development of the human placenta. *Development,* 146.
UKPDS 33. 1998. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes. *Lancet,* 352**,** 837-853.
UK Diabetes Technology Network. 2020. Best practice guide: Using diabetes technology in pregnancy v2.0.
Usman, S., Foo, L., Tay, J., Bennett, P. R. & Lees, C. 2017. Use of magnesium sulfate in preterm deliveries for neuroprotection of the neonate. *Obstetrician Gynaecologist,* 19**,** 21-28.
Valdés, G., Acuña, S., Munizaga, A., Soto, G. X. & Figueroa, C. D. 2016. Utero-placental cellular and nuclear expression of bradykinin B2 receptors in normal and preeclamptic pregnancies. *Pregnancy Hypertens,* 6**,** 30-37.
Van Der Pligt, P., Willcox, J., Szymlek-Gay, E. A., Murray, E., Worsley, A. & Daly, R. M. 2018. Associations of Maternal Vitamin D Deficiency with Pregnancy and Neonatal Complications in Developing Countries: A Systematic Review. *Nutrients,* 10.
Vanassche, F. A., Spitz, B., Hanssens, M., Vangeet, C., Arnout, J. & Vermylen, J. 1993. Increased thromboxane formation in diabetic pregnancy as a possible contributor to preeclampsia. *AJOG,* 168**,** 84-87.
Vieira, M. C., Begum, S., Seed, P. T., Badran, D., Briley, A. L., Gill, C., et al. 2018. Gestational diabetes modifies the association between PlGF in early pregnancy and preeclampsia in women with obesity. *Pregnancy Hypertens,* 13**,** 267-272.
Vitoratos, N., Valsamakis, G., Mastorakos, G., Boutsiadis, A., Salakos, N., Kouskouni, E. & Creatsas, G. 2008. Pre- and early post-partum adiponectin and Interleukin-1beta levels in women with and without gestational diabetes. *Hormones,* 7**,** 230-236.
Von Dadelszen, P., Payne, B., Li, J., Ansermino, J. M., Pipkin, F. B., Côté, A.-M., et al. 2011. Prediction of adverse maternal outcomes in pre-eclampsia: development and validation of the fullPIERS model. *Lancet,* 377**,** 219-227.
Voormolen, D. N., De Wit, L., Van Rijn, B. B., Devries, J. H., Heringa, M. P., Franx, A., et al. 2018. Neonatal Hypoglycemia Following Diet-Controlled and Insulin-Treated Gestational Diabetes Mellitus. *Diabet Care,* 41**,** 1385.

Wallis, A. B., Saftlas, A. F., Hsia, J. & Atrash, H. K. 2008. Secular Trends in the Rates of Preeclampsia, Eclampsia, and Gestational Hypertension, United States, 1987–2004. *Am J Hypertens,* 21**,** 521-526.
Wallner, W., Sengenberger, R., Strick, R., Strissel, P. L., Meurer, B., Beckmann, M. W. & Schlembach, D. 2007. Angiogenic growth factors in maternal and fetal serum in pregnancies complicated by intrauterine growth restriction. *Clin Sci,* 112**,** 51-7.
Walsh, S. W. & Coulter, S. 1989. Increased placental progesterone may cause decreased placental prostacyclin production in preeclampsia. *Am J Obstet Gynecol,* 161**,** 1586-92.
Wang, L., Chopp, M., Szalad, A., Liu, Z., Bolz, M., Alvarez, F. M., et al. 2011. Phosphodiesterase-5 is a therapeutic target for peripheral neuropathy in diabetic mice. *Neuroscience,* 193**,** 399-410.
Wang, M., Athayde, N., Padmanabhan, S. & Cheung, N. W. 2019. Causes of stillbirths in diabetic and gestational diabetes pregnancies at a NSW tertiary referral hospital. *Aust N Z J Obstet Gynaecol,* 59**,** 561-566.
Wang, Y. & Zhao, S. 2010. Chapter 2, Placental Blood Circulation. In: *Vascular Biology of the Placenta*. San Rafael (CA): Morgan & Claypool Life Sciences. Available from https://www.ncbi.nlm.nih.gov/books/NBK53254/ - last accessed 7/10/2021
Wang, Y. P., Walsh, S. W., Guo, J. D. & Zhang, J. Y. 1991. Maternal levels of prostacyclin, thromboxane, vitamin E, and lipid peroxides throughout normal pregnancy. *AJOG,* 165**,** 1690-4.
Warso, M. A. & Lands, W. E. 1983. Lipid peroxidation in relation to prostacyclin and thromboxane physiology and pathophysiology. *Br Med Bull,* 39**,** 277-280.
Waters, T. P., Dyer, A. R., Scholtens, D. M., Dooley, S. L., Herer, E., Lowe, L. P., et al. 2016. Maternal and Neonatal Morbidity for Women Who Would Be Added to the Diagnosis of GDM Using IADPSG Criteria: A Secondary Analysis of the Hyperglycemia and Adverse Pregnancy Outcome Study. *Diab Care,* 39**,** 2204.
Watson, A. J. 1992. The cell biology of blastocyst development. *Mol Reprod Devel,* 33**,** 492-504.
Wegmann, T. G., Lin, H., Guilbert, L. & Mosmann, T. R. 1993. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a Th2 phenomenon? *Immunol Today,* 14**,** 353-356.
Weissgerber, T. L., Milic, N. M., Milin-Lazovic, J. S. & Garovic, V. D. 2016. Impaired Flow-Mediated Dilation Before, During, and After Preeclampsia. *Hypertension,* 67**,** 415-423.
Weissgerber, T. L. & Mudd, L. M. 2015. Preeclampsia and diabetes. *Curr Diab Rep,* 15**,** 9.
Wilkes, B. M., Susin, M. & Mento, P. F. 1993. Localization of endothelin-1-like immunoreactivity in human placenta. *J histochem cytochem,* 41**,** 535-541.
Wolff, K., Carlström, K., Fyhrquist, F., Hemsén, A., Lunell, N.-O. & Nisell, H. 1997. Plasma Endothelin in Normal and Diabetic Pregnancy. *Diab Care,* 20**,** 653.

World Health Organisation 2013. Diagnostic Criteria and Classification of Hyperglycaemia First Detected in Pregnancy.
Wright, D., Rolnik, D. L., Syngelaki, A., De Paco Matallana, C., Machuca, M., De Alvarado, M., et al. 2018. Aspirin for Evidence-Based Preeclampsia Prevention trial: effect of aspirin on length of stay in the neonatal intensive care unit. *AJOG,* 218**,** 612.e1-612.e6.
Wu, L., Girouard, H. & De Champlain, J. 2000. Involvement of the cyclic GMP pathway in the superoxide-induced IP3 formation in vascular smooth muscle cells. *J Hypertens,* 18**,** 1057-64.
Xu, J., Zhao, Y. H., Chen, Y. P., Yuan, X. L., Wang, J., Zhu, H. & Lu, C. M. 2014. Maternal Circulating Concentrations of Tumor Necrosis Factor-Alpha, Leptin, and Adiponectin in Gestational Diabetes Mellitus: A Systematic Review and Meta-Analysis. *Sci World J,* 2014**,** 926932.
Yamamoto, J. M., Corcoy, R., Donovan, L. E., Stewart, Z. A., Tomlinson, G., Beardsall, K., et al. 2019. Maternal glycaemic control and risk of neonatal hypoglycaemia in Type 1 diabetes pregnancy: a secondary analysis of the CONCEPTT trial. *Diabet Med,* 36**,** 1046-1053.
Yamamoto, J. M., Kallas-Koeman, M. M., Butalia, S., Lodha, A. K. & Donovan, L. E. 2017. Large-for-gestational-age (LGA) neonate predicts a 2.5-fold increased odds of neonatal hypoglycaemia in women with type 1 diabetes. *Diab Metab Res Rev,* 33**,** e2824.
Yang, W., Han, F., Gao, X., Chen, Y., Ji, L. & Cai, X. 2017. Relationship Between Gestational Weight Gain and Pregnancy Complications or Delivery Outcome. *Sci Rep,* 7**,** 12531.
Yao, R., Park, B. Y., Foster, S. E. & Caughey, A. B. 2017. The association between gestational weight gain and risk of stillbirth: a population-based cohort study. *Ann Epidemiol,* 27**,** 638-644.e1.
Yildiz Atar, H., Baatz, J. E. & Ryan, R. M. 2021. Molecular Mechanisms of Maternal Diabetes Effects on Fetal and Neonatal Surfactant. *Children,* 8(4), 281
Zeisler, H., Llurba, E., Chantraine, F., Vatish, M., Staff, A. C., Sennström, M., et al. 2016. Predictive Value of the sFlt-1:PlGF Ratio in Women with Suspected Preeclampsia. *NEJM,* 374**,** 13-22.
Zhang, H., Park, Y., Wu, J., Chen, Xiu p., Lee, S., Yang, J., et al. 2009. Role of TNF-α in vascular dysfunction. *Clin Sci,* 116**,** 219-230.
Zhao, L.-P., Sheng, X.-Y., Zhou, S., Yang, T., Ma, L.-Y., Zhou, Y. & Cui, Y.-M. 2015. Metformin versus insulin for gestational diabetes mellitus: a meta-analysis. *Br J Clin Pharmacol,* 80**,** 1224-1234.
Zhu, W. W., Yang, H.X., Wei, Y.M., Yan, J., Wang, Z.L., Li, X.L., et al. 2013. Evaluation of the Value of Fasting Plasma Glucose in the First Prenatal Visit to Diagnose Gestational Diabetes Mellitus in China. *Diab Care,* 36**,** 586.
Ziauddeen, N., Wilding, S., Roderick, P. J., Macklon, N. S. & Alwan, N. A. 2019. Is maternal weight gain between pregnancies associated with risk of large-for-

gestational age birth? Analysis of a UK population-based cohort. *BMJ Open,* 9**,** e026220.
Åsvold, B. O., Vatten, L. J., Romundstad, P. R., Jenum, P. A., Karumanchi, S. A. & Eskild, A. 2011. Angiogenic Factors in Maternal Circulation and the Risk of Severe Fetal Growth Restriction. *Am J Epidemiol,* 173**,** 630-639.