Obesity and metformin in pregnancy

By Carolyn Chiswick

THE UNIVERSITY of EDINBURGH

This thesis has been submitted in fulfilment of the requirements for a postgraduate degree (e.g. PhD, MPhil, DClinPsychol) at the University of Edinburgh. Please note the following terms and conditions of use:

Abstract

Obesity is the most common antenatal comorbidity, affecting one in five of the antenatal population in the UK. It is associated with adverse outcomes for mother and baby in both the short and long term. Increasing data suggest that maternal obesity may programme offspring later life obesity and premature mortality, with high birth weight being a marker for increased risk. The mechanism by which maternal obesity causes excessive neonatal birth weight is incompletely understood but considerable evidence implicates insulin resistance and/or hyperglycaemia. There are currently no effective interventions to mitigate the effects of obesity during pregnancy. In this thesis, we present the findings from a randomised, double blind, placebo controlled trial designed to examine the efficacy of metformin, an insulin-sensitising agent, in obese pregnant women. The aim of the trial was to determine whether giving metformin to obese pregnant women from between 12 and 16 weeks' gestation until birth, would improve maternal and fetal outcomes. The primary outcome measure was birth weight of the baby, using this as a surrogate marker for the future life risk of the child developing obesity.

Nested within this large clinical trial were a series of mechanistic sub-studies. To examine the effect of metformin on maternal insulin resistance at 36 weeks' gestation, we used the hyperinsulinaemic euglycaemic clamp with concomitant use of stable isotope tracers. This enabled us to characterise in greater detail insulin sensitivity, endogenous glucose production and lipolysis. To determine the effect of metformin on maternal and fetal body composition we used magnetic resonance imaging and spectroscopy. This allowed us to quantify subcutaneous and intra-abdominal adipose tissue deposition and hepatic and skeletal muscle ectopic lipid deposition in the mother; and to measure subcutaneous adipose tissue deposition, hepatic lipid and hepatic volume in the fetus. To determine the effect of metformin on maternal endothelial function, we measured endothelium-dependent flow-mediated dilatation at the beginning and end of pregnancy. Change in diameter of the

brachial artery in response to a flow stimulus created by arterial occlusion was measured using ultrasound imaging.

We found no significant effect of metformin on birth weight. Mean birth weight was 3463 g (SD 660) in the placebo group and 3462 g (SD 548) in the metformin group (adjusted mean difference in z score –0·029, 95% CI –0·217 to 0·158; p=0·7597). Subjects taking metformin did demonstrate increased insulin sensitivity (M/I difference between means during high dose insulin of 0.02 [95% CI 0.001 to 0.03] milligrams per kilogram fat free mass per minute per pmol/L, p=0.04) but also enhanced endogenous glucose production (difference between means 0.54 [95% CI 0.08 to 1.00] milligrams per kilogram fat free mass per minute, p=0.02), compared with those taking placebo. We did not demonstrate any differences between treatment groups in maternal subcutaneous and intra-abdominal adipose tissue, or ectopic lipid deposition, or in fetal body fat distribution and liver volume. Participants in both treatment groups demonstrated a decline in endothelium-dependent flow-mediated dilatation between early and late pregnancy but there were no differences in the magnitude of that decline between the treatment groups.

In conclusion, metformin, administered to obese, non-diabetic pregnant women, does not have any significant effect on birth weight of the baby. Our clamp studies demonstrated that subjects taking metformin were indeed more insulin-sensitive than those taking placebo, but the higher endogenous glucose production in this group suggests a reduced ability to suppress hepatic glucose production in response to insulin. This increased glucose flux may in part explain the lack of effect of metformin on fetal nutrition and growth. We can conclude that metformin, should not be used as an intervention in obese pregnant women to prevent excess birth weight. The global obesity epidemic is one of the greatest public health challenges we face and the cycle of disadvantage continues to be perpetuated to the next generation. The lack of any effective interventions for this high-risk group remains a significant concern and an important area for further research.

Lay Summary

Obesity during pregnancy is common. This is of concern because obese women have an increased risk of complications including diabetes and pre-eclampsia. There is also an increased risk for their babies to be born larger than average, or to be stillborn. In addition, there may be harmful effects of maternal obesity that persist into the baby's adult life, including a higher risk of obesity and premature death.

We don't know how obesity causes these problems. We do know that obese pregnant women have higher blood glucose and respond less well to the hormone insulin than lean pregnant women, i.e. they are 'insulin resistant'. This means that the food supply to the baby is potentially too great, leading to a higher birthweight. The link between insulin resistance and high birthweight has already been demonstrated, as has a link between high blood glucose and greater risk of pregnancy problems.

The aim of this study was to see whether giving obese pregnant women a drug called metformin reduced the risk of them having a larger than average baby. Metformin is safe to take during pregnancy and works by reducing insulin resistance.

We recruited 449 women to take part in the study. They were randomly assigned to receive treatment with either metformin or placebo tablets during their pregnancy. A subgroup of the women participating in the study took part in some extra experiments to examine the effect of metformin on insulin resistance in greater detail. Some participants also had magnetic resonance scans to examine the effect of metformin on fat distribution in the body and in the developing fetus. Additionally, some participants had an extra test to look at the effect of metformin on blood vessel function.

The results showed the average birthweight of babies born to women in both groups was similar, 3463 g in the placebo group and 3462 g in the metformin group. There

was no increased risk of a bad outcome in either of the groups with the exception of nausea and vomiting which were more common in the metformin group. In the more detailed tests, we found that metformin did make the body slightly less insulin resistant but there was no overall effect on blood glucose. There was no effect of metformin on body fat distribution in the mother or baby, or on blood vessel function.

We can conclude that metformin is not an effective treatment for obese pregnant women to reduce the risk of having a larger than average baby.

Declaration

I declare that this thesis has been composed by me and the work described in this thesis has not previously been accepted for, nor is currently being submitted in candidature for another degree. I confirm that the work submitted is my own, except where work which has formed part of jointly-authored publications has been included. My contribution and those of the other authors to this work have been indicated below. I confirm that appropriate credit has been given within this thesis where reference has been made to the work of others.

The work presented in **Chapter 2** was previously published in *BMJ Open* as Efficacy of metformin in pregnant obese women: a randomised controlled trial by **Carolyn Chiswick**, Rebecca M Reynolds, Fiona C Denison, Sonia A Whyte, Amanda J Drake, David E Newby, Brian R Walker, Shareen Forbes, Gordon D Murray, Siobhan Quenby, Susan Wray and Jane E Norman.

Jane Norman conceived and designed the study. Jane Norman, Brian Walker, David Newby, Fiona Denison, Rebecca Reynolds, Amanda Drake, Shareen Forbes, Gordon Murray, Siobhan Quenby and Sonia Whyte drafted the original grant proposal and trial protocol. Gordon Murray provided methodological and statistical expertise. David Newby provided expertise in the vascular studies. Jane Norman and Fiona Denison provided expertise in maternal clinical outcomes. Amanda Drake provided expertise in neonatal outcomes. Siobhan Quenby and Susan Wray provided expertise on myometrial contractility. Sonia Whyte drafted the original protocol and is the trial manager. **Carolyn Chiswick** wrote the manuscript and was responsible for the day-to day running of the trial included participant recruitment, data collection and liaising with sites. **Carolyn Chiswick** was also responsible for carrying out the body composition, MRI, endothelial function and hyperinsulinaemic euglycaemic clamp substudies.

The work presented in **Chapter 3** was previously published in *The Lancet Diabetes and Endocrinology* as Effect of metformin on maternal and fetal outcomes in obese pregnant women (EMPOWaR): a randomised, double-blind, placebo-controlled trial by **Carolyn Chiswick**, Rebecca M Reynolds, Fiona Denison, Amanda J Drake, Shareen Forbes, David E Newby, Brian R Walker, Siobhan Quenby, Susan Wray, Andrew Weeks, Hany Lashen, Aryelly Rodriguez, Gordon Murray, Sonia Whyte and Jane E Norman.

Jane Norman conceived the study and drafted the manuscript. Jane Norman, Rebecca Reynolds, Fiona Denison, Amanda Drake, Shareen Forbes, David Newby, Brian Walker, Siobhan Quenby, Susan Wray, Gordon Murray and Sonia Whyte designed the study. **Carolyn Chiswick**, Jane Norman, Rebecca Reynolds, Fiona Denison, Amanda Drake, Shareen Forbes, Siobhan Quenby, Andrew Weeks, Hany Lashen and Susan Wray acquired the data. Gordon Murray and Aryelly Rodriguez analysed the data. All authors interpreted the data, revised the paper critically for important intellectual content, and approved the final version.

The work presented in the **appendix** has been published by *Efficacy and Mechanism Evaluation, National Institute for Health Research Journals Library* as Efficacy of metformin in pregnant obese women, a randomised controlled trial by **Carolyn A Chiswick**, Rebecca M Reynolds, Fiona C Denison, Amanda J Drake, Shareen Forbes, David E Newby, Brian R Walker, Siobhan Quenby, Susan Wray, Andrew Weeks, Hany Lashen, Aryelly Rodriguez, Gordon D Murray, Sonia Whyte, Ruth Andrew, Natalie Homer, Scott Semple, Calum Gray, Marian C Aldhous, Karen Noble, Sarah Cunningham-Burley, Alice Keely and Jane E Norman.

Jane Norman conceived the study. Jane Norman, Rebecca Reynolds, Fiona Denison, Amanda Drake, Shareen Forbes, David Newby, Brian Walker, Siobhan Quenby, Sonia Whyte, Susan Wray and Gordon Murray designed the study. **Carolyn Chiswick**, Jane Norman, Rebecca Reynolds, Fiona Denison, Amanda Drake, Shareen Forbes, David Newby, Brian Walker, Siobhan Quenby, Andrew Weeks,

Acknowledgements

Contents

List of tables and figures

xv

Abbreviations

ACCORD	Academic and Clinical Central Office for Research and Development
ADP	air displacement plethysmography
ACE	angiotensin converting enzyme
AE	adverse event
ALT	alanine aminotransferase
AR	adverse reaction
AUC	area under the curve
BMI	body mass index
CRP	C-reactive protein
DEXA	dual-energy X-ray absorptiometry
DNA	deoxyribonucleic acid
eCRF	electronic case report form
ECTU	Edinburgh Clinical Trials Unit
EGP	endogenous glucose production
ELISA	enzyme linked immunosorbent assay
EMPOWaR	Efficacy of Metformin in Pregnant Obese Women, a Randomised controlled trial
FFM	fat-free mass
FMD	flow-mediated dilatation

GCP	good clinical practice
GDM	gestational diabetes mellitus
GR	glucocorticoid receptor
HDL	high-density lipoprotein
HEC	hyperinsulinaemic euglycaemic clamp
HOMA-IR	homeostatic model assessment – insulin resistance
HPA	hypothalamic-pituitary-adrenal
HSD	hydroxysteroid dehydrogenase
IADPSG	International Association of the Diabetes in Pregnancy Study Groups
IL	interleukin
ITT	intention to treat
IR	insulin resistance
ITT	intention to treat
LDL	low-density lipoprotein
LGA	large for gestational age
LiP	Lifestyle in Pregnancy
M/I	glucose disposal per unit plasma insulin
MOP	Metformin in Obese Pregnancy
MRC	Medical Research Council
MRI	magnetic resonance imaging

MRS	magnetic resonance spectroscopy
NEFA	non-esterified fatty acid
NHS	National Health Service
NICE	National Institute for Health and Clinical Excellence
OR	odds ratio
PAI	plasminogen activator inhibitor
PCOS	polycystic ovary syndrome
PSS	physiological saline
PP	per protocol
Ra	rate of appearance
RCT	randomised controlled trial
Rd	rate of disappearance
RNA	ribonucleic acid
RT	reverse transcriptase
SAE	serious adverse event
SD	standard deviation

SIGN   Scottish Intercollegiate Guidelines Network

SOP   standard operating procedure

UPBEAT   UK Pregnancies Better Eating and Activity Trial

WGD   whole-body glucose disposal

WHO   World Health Organisation

1. Chapter 1   Introduction

1.1. Background

The World Health Organisation (WHO) defines overweight or obesity as abnormal or excessive fat accumulation that may impair health[1]. The diagnosis of obesity is commonly made on the basis of a body mass index (BMI: weight in kilograms divided by the square of the height in meters, kg/m^2) exceeding a defined threshold, usually 30 kg/m^2. Obesity is a chronic disease with a multifactorial aetiology that leads to serious health consequences. Well-established contributory causes include genetic, environmental and behavioural factors but there is now mounting evidence that the intra-uterine environment to which we are exposed also plays an important role in our risk of developing obesity.

Rates of obesity have risen alarmingly in recent decades[2]. The majority of the world's population now live in countries where overweight and obesity is responsible for more deaths than underweight[3]. In general, men have higher rates of overweight (BMI greater than 25 kg/m^2) but women have higher rates of obesity. In common with global trends, around 20% of women booking for antenatal care in the UK are obese, making it the commonest antenatal comorbidity. The adverse effects of maternal obesity on pregnancy complications for both the mother and fetus are well established and cover every aspect of pregnancy from pre-conception to the puerperium. For the fetus, there is a greater risk of miscarriage[4], and fetal abnormality[5], iatrogenic preterm birth[6] and a one- to five-fold increased risk of stillbirth[7, 8]. During her pregnancy, an obese woman has a two-fold increased risk of developing pre-eclampsia[9] and a three to four fold increased risk of venous thromboembolism[10] and gestational diabetes[11]. Around the time of delivery, induction of labour, caesarean section, postpartum haemorrhage and infection are all more likely[11-13]. Finally, a recurring theme of the confidential enquiries into maternal deaths of recent decades has been obesity[14].

In addition to these immediate complications, there is mounting evidence of a detrimental effect on the longer-term health of offspring of women who are obese during pregnancy[15-17]. Increasingly, data suggest that maternal obesity may programme offspring later life obesity, with high birthweight being a marker for increased risk. Our own recent work also suggests that offspring of obese pregnant women are at increased risk of premature death in adulthood[18].

1.2. Evidence for mechanisms linking maternal obesity to offspring obesity

The mechanism by which maternal obesity causes excessive neonatal birthweight and later life ill health is not clearly understood. As previously mentioned, obesity is a multifactorial disease and unpicking the relative contributions of genetics, environment, behaviour and programming or epigenetics continues to be a challenging area of research.

The 'developmental overnutrition hypothesis' was first proposed in the 1950s by Pederson to explain the association between maternal diabetes and excessive growth of the fetus[19]. Pederson postulated that the greater delivery of glucose to the fetus resulted in fetal hyperinsulinaemia in order for the fetus to manage its hyperglycaemia. This in turn caused insulin-mediated excessive fetal growth. This hypothesis has subsequently been broadened to include other fuel sources that may contribute to fetal hyperinsulinaemia and excessive growth such as free fatty acids and amino acids and more recently to include exposure to obesity in general as a risk[20, 21]. A number of animal studies support the hypothesis that maternal obesity has a permanent impact on offspring obesity, body composition and cardiometabolic health, along with an increasing number of human studies.

1.2.1. Evidence from animal studies

Timing of exposure to the insult appears to be important in the resulting offspring phenotype. In most rodent studies of maternal obesity, animals are fed an obesogenic diet until significantly heavier than control animals, and then continue on

the same diet throughout pregnancy and lactation. This means the effect of obesity cannot be separated from that of overnutrition. However, there may be particular windows of development during which maternal obesity has a detrimental effect. There are some rodent data to suggest that maternal obesity impairs oocyte quality and thus development of the early embryo, suggesting that programming effects in the offspring occur even before fertilisation[22]. Other studies have employed cross-fostering techniques that demonstrate the importance of overnutrition purely during pregnancy[23], while some suggest diet during pregnancy and lactation is important[24-26].

In addition to the programming effects of maternal obesity on offspring obesity, it seems there is also an impact on body composition, which may impact on lifelong cardiometabolic health. Rat offspring of mothers fed a junk food diet during pregnancy or both pregnancy and lactation exhibited increased intramuscular lipid content with altered gene expression of genes important for muscle growth and metabolism[27, 28]. Similar changes have also been demonstrated in sheep exposed to maternal obesity, in association with increased expression of inflammatory markers and altered AMP-activated protein kinase signalling[29-31]. This could have an impact on muscle size and strength in later life, and therefore ability to keep physically active. Additionally, intramuscular lipid and altered gene expression is likely to be important in the development of peripheral insulin resistance. Indeed, one study demonstrated that offspring of obese mice exhibit changes in insulin signalling and muscle mitochondrial complex activity in early adulthood[32].

A further extension of the developmental overnutrition hypothesis encompasses some evidence that programming of appetite control and neuroendocrine function may also occur. For example, offspring of mice fed a highly palatable diet during pregnancy and lactation demonstrate hyperphagia prior to the development of obesity[33], and rat offspring exposed to a junk food diet in pregnancy develop a preference for fatty, sugary, salty foods compared to control animals[34]. This may reflect programmed changes to the hypothalamus, which is important for regulation

of appetite. Furthermore, one study in sheep in which direct infusions of glucose were administered into the fetuses in late gestation found altered expression of orexigenic peptides in the fetal hypothalamus, suggesting prenatal overnutrition may alter the brain sufficiently to impact on appetite regulation[35].

1.2.2. Evidence from human studies

The alarming rise in maternal obesity rates has been accompanied by a rise in babies born large for gestational age[36] and childhood obesity, including very early onset of obesity in the first six months of life[37, 38]. Maternal obesity is undoubtedly associated with fetal overgrowth[39] and there are numerous studies which link high birthweight with an increased risk of obesity in later life[17]. There are several observational and cohort studies which demonstrate an association between maternal obesity and effects on offspring adiposity at all stages in life from infancy, through childhood and into adulthood[40-44]. These studies and others lend strong support to the overnutrition hypothesis but do not really provide mechanistic evidence of an effect of obesity itself, rather than, for example metabolic disturbance commonly associated with obesity such as gestational diabetes. Many are based on historical cohorts that may rely on weight or BMI data being self-reported or in cohorts where the prevalence of obesity was much less than it is today. The evidence of a causal effect of obesity itself is more compelling at the extreme of obesity. For example, Smith *et al* examined offspring of women who had undergone bariatric surgery between pregnancies and showed offspring after maternal weight loss to be leaner, with greater insulin sensitivity and more a favourable lipid profile[45].

Gestational weight gain (as opposed to obesity *per se*) is positively associated with offspring obesity in childhood and adulthood[46-51]. Human studies specifically addressing dietary components in the context of maternal overnutrition are difficult to perform but the degree of gestational weight gain may be a reflection of the nutritional environment to which the fetus is exposed. However, interpretations of the findings of most of these studies is again limited as many use self-reported pre-pregnancy BMI and do not have detailed measurements of maternal weight and body

composition during pregnancy. It remains unclear whether gestational weight gain is a causal intrauterine mechanism. It is a multifactorial risk and reflects a number of different components (e.g. various maternal fat depot sites, fetal weight, placental weight) and only by separating these out (e.g. with detailed imaging in pregnancy) would it be possible to interpret the findings fully.

1.2.3. Epigenetics

Epigenetics, the study of how changes in gene activity during development cause specific phenotypes to emerge over the lifetime of an individual[52], has been strongly implicated in the developmental origins of health and disease. The large and rapid increase in the worldwide prevalence of obesity cannot be explained by changes in the genome. Human epigenetic research to date has primarily focused particularly on maternal under-nutrition but has demonstrated that epigenetic changes mediated by the maternal environment can be transmitted to subsequent generations[53]. Animal studies have shown that maternal diet can induce epigenetic changes in offspring and cause long-term alterations in gene expression of genes involved in obesity[54]. For example, in primates exposure to a high fat diet has been associated with global as well as gene-specific alterations in DNA methylation and histone modifications[55]. In a human study, maternal hyperglycaemia was shown to correlate with placental leptin gene DNA methylation levels[56]. More recently, consistent methylation changes in genes closely associated with adult obesity have been demonstrated[57]. Such epigenetic processes may well be part of the mechanism underlying developmental overnutrition but this is young field of research and findings must be replicated before robust conclusions can be drawn.

1.2.4. Insulin resistance

Considerable evidence implicates insulin resistance and/or hyperglycaemia as the causal mechanism by which maternal obesity cause excessive neonatal birthweight. Pre-existing maternal diabetes, particularly with poor glycaemic control, has long been recognised to be associated with poor obstetric outcomes and fetal macrosomia.

More recently, several studies have demonstrated that treating hyperglycaemia at levels below those associated with overt diabetes outwith pregnancy, reduces the risk of adverse pregnancy outcome. The Australian Carbohydrate Intolerance Study in Pregnant women trial group (ACHOIS) published a randomised controlled trial of treating mild hyperglycaemia in pregnant women who did not reach existing diagnostic criteria for gestational diabetes[58]. The investigators found the treatment group demonstrated a reduction in the composite endpoint of perinatal death, shoulder dystocia, bone fracture and nerve palsy, all of which are associated with macrosomia. Similarly a trial by Landon *et al* demonstrated a reduction in macrosomia, shoulder dystocia, caesarean delivery and hypertensive disorders in women treated for mild hyperglycaemia[59]. In 2008, the landmark trial The Hyperglycaemia and Adverse Pregnancy Outcomes (HAPO) study was published[60]. This was a multinational, prospective, blinded, observational study of almost 25 000 pregnant women. It was designed to evaluate the relationship between maternal glucose concentrations and adverse pregnancy outcome. HAPO confirmed that there was a linear relationship between hyperglycaemia and birthweight, with no clear threshold above which adverse events occurred.

Although these informative studies were not restricted to obese pregnant women, we do know that obese pregnant women are more insulin resistant and hyperglycaemic than their lean counterparts[62]. This enhances nutrient availability for the fetus with consequent excessive growth. For example, there is strong correlation between the degree of insulin resistance in late pregnancy and both birthweight and fat-free mass at birth[63]. It is logical to assume that this enhanced exposure to hyperglycaemia of the developing fetus of an obese mother is a plausible causative mechanism of excessive fetal growth and subsequent life long risk of obesity. An intervention in pregnancy in obese women to improve insulin sensitivity and thus reduce fetal exposure to hyperglycaemia may well reduce the risk of fetal overgrowth. Lifestyle modification, primarily in the form of dietary modification and exercise are possible candidates for such an intervention and the outcome of trials of such interventions are discussed below. An alternative to these strategies is pharmacotherapy. Metformin is an insulin sensitising agent, which is safe for use in pregnant women.

This thesis describes the findings from a multicentre, double-blind, randomised-controlled trial of metformin versus placebo in obese pregnant women with the primary outcome of birthweight, and a number of nested substudies to examine the mechanism of any effect of treatment.

1.3. Interventions in pregnancy to reduce excess birthweight in offspring of obese pregnant women

To date, all of the interventions that have been trialled in overweight or obese pregnant women to reduce the risk of excess birthweight in the offspring have involved modifications to diet, lifestyle or a combination of both.

There have been several systematic reviews of studies evaluating such interventions in pregnancy but only two have been limited to overweight and obese women, Dodd et al in 2010[64] and Oteng-Ntim et al in 2012[65]. Three further randomised trials have been published since these reviews, the LIMIT trial (Limiting weight gain in overweight and obese women during pregnancy to improve health outcomes: the LIMIT randomised controlled trial)[66], the LiP (Lifestyle in Pregnancy) Study[67], and UPBEAT (UK Pregnancies Better Eating and Activity trial)[68].

The review by Dodd et al examined nine randomised controlled trials, including 743 women. Seven trials compared a dietary intervention with standard antenatal care. Two of the studies evaluated the effect of an exercise intervention but outcomes did not include effect on infant birthweight in these studies. Only three trials reported outcome data for the primary outcome large-for-gestational-age (LGA) infants, with no significant difference between those who received the intervention and those who did not (366 women; risk ratio 2.20; 95% CI 0.84 to 4.86). Four studies examined effect on gestational weight gain and again there was no statistically significant difference in this outcome (416 women; weighted mean difference -3.10kg; 95% CI -

7

8.32 to 2.13). The overall conclusion of the review was that the evidence of benefit for this type of intervention in overweight or obese women is not clear. However, the authors note the quality of all the included studies was poor to fair and further high quality, suitably powered randomised trials are urgently needed.

The review by Oteng-Ntim *et al* included 13 randomised trials and six non-randomised trials. Again, overall quality of the trials was deemed to be suboptimal, with five of the RCTs judged to be of medium quality and the rest of low quality. Six of the studies included large-for-gestational-age as an outcome, but there was no evidence that the interventions were associated with a lower prevalence of this outcome (1008 women, OR 0.91; 95% CI 0.62 to 1.32). There were seven studies that examined effect on birthweight and although there was a trend towards an effect of the intervention, this did not reach statistical significance (1133 women, mean difference -56.64; 95% CI -120.15 to 6.88). The authors reach a similar conclusion that further meta-analyses will be unlikely to refine the quality of the evidence and that large-scale suitably powered trials are required.

One such trial has since been published – the LIMIT trial[66]. This was a multicentre randomised controlled trial of a diet, exercise and behavioural intervention versus standard care for overweight or obese women (BMI>25 kg/m^2, median BMI of cohort 31.1 kg/m^2). The primary outcome was infants born large-for-gestational-age (>90th centile for gestation). The trial recruited to target a total of 2212 women and was adequately powered to detect a 30% reduction in LGA infants. There was no significant difference in the risk of infants born LGA in the lifestyle advice group, compared with standard care group (19% versus 21%; adjusted relative risk 0.90; 95% CI 0.77 to 1.07, p=0.24).

The LiP (Lifestyle in Pregnancy) Study[67] was a smaller trial of 360 women, all of who were obese (BMI 30-45 kg/m^2, median BMI 33kg/m^2). They were randomised to receive a lifestyle intervention, which included dietetic advice, gym membership, physical training and personal coaching. The primary end-point was a combination

of five obstetric and neonatal outcomes: emergency caesarean section, pre-eclampsia, GDM, LGA and admission to the neonatal unit, with a score of 1 point for each outcome. There was no significant difference in combined scores (0.65 for the intervention, versus 0.67 for the control group, p=0.39). Birthweight was in fact significantly higher in the intervention group (median 3742g vs 3596g, p=0.039). Gestational weight gain was significantly lower in the intervention group (7.0kg vs 8.6kg; p=0.01). However, as with many of the previous studies, the authors note that ultimately they were underpowered, with power calculations being based on the expectation of a larger difference in gestational weight gain between groups than was actually found.

The UPBEAT study similarly found no effect of a lifestyle intervention on the incidence of gestational diabetes nor large for gestational age infants[68].

At the time of initiation of the EMPOWaR study, there were no randomised controlled trials of pharmacotherapy as an intervention for obese pregnant women. Given the evidence of a lack of effect from lifestyle interventions, pharmacotherapy is an important next step. Other than the work presented in this report, we are aware of two other on-going studies of the effect of metformin as a pharmacological intervention in obese pregnant women (MOP: NCT01273584 and GRoW:ACTRN12612001277831), one of which has now been published[69].

1.4. Metformin

Galega officinalis, the French lilac, was prescribed in medieval Europe to relieve the intense urination associated with what has since become known as diabetes mellitus [70]. The hypoglycaemic properties of the active ingredient in the plant, guanidine, have long been recognised. Metformin, a synthetic biguanide (two linked guanidine rings), was developed in the 1920s and used for many years in Europe to treat diabetes prior to being approved by the FDA in the USA in the mid 1990s for use in type 2 diabetes. It is now the most commonly prescribed oral anti-diabetic drug

world wide, taken by 150 million people each year. And yet, its actual mechanism of action remains incompletely understood.

Metformin is not metabolised. It accumulates in the liver following absorption through the intestinal epithelium and transfer via the portal vein. The liver is its primary site of action. It is excreted unchanged by the kidneys.

Metformin acts primarily to suppress hepatic glucose production but it also increases peripheral glucose utilisation and inhibits adipose tissue lipolysis. The exact molecular locus of metformin's effects remains unclear but its action on AMPK activation seems to play a central role[71]. Its effect on glucose metabolism appears to be mediated via its action on the mitochondrial respiratory chain and consequent activation of AMPK. Metformin inhibits the mitochondrial respiratory complex I leading to reduced ATP production and activation of AMPK[72, 73]. In the liver, the activation of AMPK decreases lipid synthesis and suppresses gluconeogenesis in hepatocytes, resulting in decreased circulating insulin and glucose. However, metformin also has AMPK-independent effects on glucose metabolism as mice deficient in AMPK demonstrate metformin-induced inhibition of glucose production[74].

1.5. Metformin in pregnancy

The use of metformin is endorsed by the National Institute for Health and Care Excellence (NICE) for the treatment of gestational diabetes (GDM)[75]. There are no placebo-controlled randomised trials of the use of metformin in pregnancy, but several trials have compared metformin with alternative agents for the treatment of GDM. There have been several recent systematic reviews of these trials including those by Balsells *et al*, 2015[76] and Zhao *et al*, 2015[77], and a literature review by Singh *et al*, 2015[78]. Additionally, two other randomised trials, Beyou *et al*, 2015[79] and George *et al*, 2015[80] have been published since these meta-analyses were performed.

The meta-analysis by Balsalls *et al*[76] compared metformin with insulin and with glibenclamide for the treatment of GDM. Fourteen primary outcomes were considered. Compared with insulin, metformin reduced maternal weight gain (mean difference −1.14 [95% CI −2.22 to −0.06] kg), reduced gestational age at delivery (mean difference −0.16 [95% CI −0.30 to −0.02] weeks), and increased preterm birth (risk ratio 1.50 [95%CI 1.04 to 2.16]). Compared with glibenclamide, metformin reduced maternal weight gain (mean difference −2.06 [95% CI −3.98 to −0.14] kg), was associated with lower birth weight (mean difference −209 [95% CI −314 to −104] kg), reduced the risk of macrosomia (risk ratio 0.33 [95% CI 0.13 to 0.81]), and reduced the risk of large for gestational age newborns (risk ratio 0.44 [95% CI 0.21 to 0.92]). Zhao *et al*[77] demonstrated that, compared with insulin, metformin reduced the risk of pregnancy-induced hypertension (risk ratio 0.54 [95% CI 0.31 to 0.91]), but there were no differences in effects on neonatal hypoglycemia, large-for-gestational age infants, respiratory distress syndrome, phototherapy or perinatal death.

The literature review[78] reported that the majority of studies found no difference in glycaemic control between metformin and insulin groups, and suggested that although there is a growing body of evidence to suggest a role for metformin in GDM management, much of this was from single site small studies, and that further studies are needed to inform guidelines.

In one of the randomised trials not included in the systematic reviews above, and which recruited 159 women, metformin was demonstrated to be superior to glibenclamide because it was associated with a reduction in risk of 16.1% (95% CI 2.5% to 29.7%; *p*=0.02) in the primary outcome, a composite of macrosomia, hypoglycaemia, need for phototherapy, respiratory distress, stillbirth or neonatal death and birth trauma, largely due to a higher incidence of hypoglycaemia in the glibenclamide group[81]. In the RCT by Beyuo *et al*[79] (n=104), which compared metformin with placebo, with the addition of insulin if required to maintain

glycaemic control, postprandial glucose levels were significantly lower in the metformin group.

There are few randomised trials of metformin versus placebo in pregnant women without GDM. Both published studies were of women with polycystic ovary syndrome[82, 83], with one being a pilot of the other. Although a significant difference in a composite of severe pregnancy and postpartum complications was seen in the smaller study comparing metformin 850mg twice daily with placebo (n=40)[82], there were no significant differences in the outcomes of pre-eclampsia, preterm delivery or gestational diabetes in the larger study comparing metformin 2000mg daily of metformin with placebo (n=259)[83], although women in the metformin group gained less weight.

1.6. Hypotheses and aims

The work in this thesis presents the full trial protocol and findings from the EMPOWaR trial (Efficacy of metformin in pregnant obese women; a randomised controlled trial). This was a double-blind randomised-controlled trial of metformin versus placebo given to obese pregnant women between 12 and 16 weeks' gestation until delivery.

We hypothesised that metformin administered to obese women during pregnancy would reduce birth weight centile in their babies and consequently their future life risk of obesity and metabolic syndrome.

A number of nested substudies were included to test the following hypotheses:

- Participants taking metformin, compared to those taking placebo, would demonstrate improved insulin sensitivity, as measured by the hyperinsulinaemic euglycaemic clamp.

- Improving insulin sensitivity with metformin would result in less deposition of lipid in the more insulin sensitive sites (i.e. visceral, hepatic and skeletal muscle), as measured using magnetic resonance imaging techniques.

- Fetuses' of mothers exposed to metformin in pregnancy would accumulate less excess fat, compared to those exposed to placebo, as measured by magnetic resonance imaging techniques.

- Improving insulin sensitivity in obese pregnant women with metformin would result in improved endothelial function in late pregnancy, as measured by flow-mediated dilatation.

The primary aim of the trial was to determine whether metformin could reduce future life risk of obesity in the baby. We used birth weight centile as a surrogate marker for future life events as its predictive value has been shown in large epidemiological studies[84].

The aims of the nested substudies were as follows:

- To measure hepatic and peripheral insulin sensitivity and rates of lipolysis in obese pregnant women, taking either metformin or placebo, at 36 weeks' gestation.

- To quantify and compare fat distribution in obese pregnant women exposed to either placebo or metformin in early and late third trimester.

- To quantify and compare hepatic and skeletal muscle lipid in obese pregnant women exposed to either placebo or metformin in early and late third trimester.

- To optimise an MR imaging protocol for measurement of fetal liver volume, fetal subcutaneous fat and fetal hepatic lipid deposition.

- To quantify and compare fetal liver volume, fetal subcutaneous fat and fetal hepatic lipid in fetuses of obese women exposed to metformin or placebo during pregnancy.

- To optimise the technique of FMD for use in obese pregnant women.

- To measure FMD in early and late pregnancy in obese pregnant women participating in the EMPOWaR trial.

- To compare endothelial function in obese pregnant women taking metformin with those taking placebo and with an additional group of lean pregnant controls.

2. Chapter 2   Protocol for Efficacy of Metformin in Pregnant Obese Women: a Randomised controlled trial (EMPOWaR)

This chapter details the full protocol for the EMPOWaR trial and was published in BMJ Open, prior to completion of the clinical trial in accordance with Consolidated Standards of Reporting Trials (CONSORT) guidance[85], as Chiswick CA, Reynolds RM, Denison FC, *et al*. Efficacy of metformin in pregnant obese women: a randomised controlled trial. *BMJ Open* 2015;**5**:e006854.doi:10.1136/bmjopen-2014-006854

We made some changes to the protocol after recruitment began, but before generation of the statistical analysis plan, publication of the protocol as detailed in this chapter, and unblinding and analysis. A full summary of all protocol changes is provided in the full trial report, which is included as an appendix to this thesis.

2.1. Abstract

Introduction

Increasing evidence suggests obesity has its origins prior to birth. There is clear correlation between maternal obesity, high birth weight and offspring risk of obesity in later life. It is also clear that women who are obese during pregnancy are at greater risk of adverse outcomes, including gestational diabetes and stillbirth. The mechanism(s) by which obesity causes these problems is unknown, although hyperglycaemia and insulin resistance are strongly implicated. We present a protocol for a study to test the hypothesis that metformin will improve insulin sensitivity in

obese pregnant women, thereby reducing the incidence of high birth weight babies and other pregnancy complications.

Methods and analysis

The Efficacy of Metformin in Pregnant Obese Women, a Randomised controlled (EMPOWaR) trial is a double-masked randomised placebo-controlled trial to determine whether metformin given to obese (BMI >30 kg/m^2) pregnant women from 12-16 weeks' gestation until delivery reduces the incidence of high birth weight babies. A secondary aim is to test the mechanism(s) of any effect. Obese women with a singleton pregnancy and normal glucose tolerance will be recruited prior to 16 weeks' gestation and prescribed study medication, metformin or placebo, to be taken until delivery. Further study visits will occur at 28 and 36 weeks' gestation for glucose tolerance testing and to record anthropometric measurements. Birth weight and other measurements will be recorded at time of delivery. Anthropometry of mother and baby will be performed at 3 months post-delivery. As of January 2014, 449 women had been randomised across the UK.

Ethics and dissemination

The study will be conducted in accordance with the principles of Good Clinical Practice. A favourable ethical opinion was obtained from Scotland A Research Ethics Committee, reference number 10/MRE00/12. Results will be disseminated at conferences and published in peer-reviewed journals.

Trial registration number

ISRCTN51279843

Strengths and limitations of this study

- This is the first multicenter, double-masked, randomised-controlled trial to examine the effect of metformin in obese pregnant women without diabetes.
- We will use a recognised surrogate (birthweight centile) as a marker of future life risk of obesity in the offspring.
- Follow-up of the offspring is limited to the early postnatal phase.

2.2. Background

Rates of obesity, as defined by a BMI greater than 30 kg/m^2, are rising alarmingly in the United Kingdom and throughout the world. Around 27% of adults in Scotland are now obese and 32% of Scottish children have a weight outwith a healthy range for their age[86]. Obesity rates in England[87] and the United States[88] are similar. The increase in average BMI during pregnancy over the last twenty years has been well documented[89]. Rates of maternal obesity in women booking for antenatal care in the United Kingdom are now around 20%. There has also been a substantial increase in mean birth weight and in the incidence of being born large for gestational age over the past few decades[36]. The secular rise in maternal weight at delivery appears to be the factor most strongly correlated with the increase in birth weight[36, 62]. Positive correlations have also been shown between birth weight and both maternal pre-gravid weight[90-92] and weight gain in pregnancy[93], with odds of high birth weight some 2.4 times greater in morbidly obese compared with lean women[94].

This increase in mean birth weight is of concern because it is linked to an increased likelihood of child and adult obesity. A systematic review showed a positive correlation between birth weight and obesity during adulthood[17]. The association between high birth weight and later life obesity is also supported by two large epidemiological studies[15, 16], both of which found obesity to be a long-term correlate of high birth weight. A subsequent prospective study found that children who were large for gestational age at birth and exposed to an intrauterine environment of maternal obesity are also at increased risk of developing metabolic syndrome[84]. Our own recent work suggests that offspring of obese pregnant women are at increased risk of premature death in adulthood[18]. Thus, the increase in rates of maternal obesity are setting up a vicious cycle, leading to increased birth weight and increased rates of child and adult obesity, contributing to an epidemic of obesity which has become one of the most significant contributors to global ill health.

Women who are obese during pregnancy are also themselves at greater risk of a number of adverse outcomes, including, gestational diabetes[11], pre-eclampsia[9] and stillbirth[7, 8]. The mechanism(s) by which maternal obesity increases pregnancy and peripartum complications are unclear, but there are likely several candidate mechanisms including hyperglycaemia and increased insulin resistance.

Clearly an intervention is urgently required. Lifestyle interventions (e.g. diet and exercise) are a logical approach and are currently being trialled, for example, UK Pregnancies Better Eating and Activity Trial (UPBEAT), Poston (ISRCTN89971375) although studies hitherto have shown no evidence of benefit[66, 95, 96]. A recent meta-analysis of dietary and lifestyle interventions suggests dietary interventions may be of some benefit over physical activity or mixed interventions in reducing maternal weight gain. However, there was no effect on birth weight, the incidence of large for gestational age babies, and clinically important adverse outcomes[97].

In this study, we test the hypothesis that the insulin-sensitising agent metformin reduces birth weight centile in the offspring of obese pregnant women. The principal action of metformin includes improvement of insulin resistance in liver and skeletal muscle, along with improved endothelial function, increased peripheral uptake of glucose, improved lipid profile, redistribution of fat from visceral to subcutaneous depots and antioxidant effects[98], all of which are likely to contribute to its clinical efficacy in reducing adverse outcomes in obese pregnant women.

Considerable evidence implicates insulin resistance or hyperglycaemia as the mechanism by which maternal obesity causes excessive neonatal birth weight. Whilst modest insulin resistance is physiological in pregnancy and generates maternal glucose, free fatty acids and amino acids as substrates for fetal growth, obese pregnant women are more insulin resistant than their lean counterparts[62] leading to a further amplification of nutrient availability with consequent excessive

fetal growth. There is a strong correlation between insulin resistance in late gestation and both birth weight and fat-free mass at birth[63]. The Hyperglycaemia and Adverse Pregnancy Outcomes (HAPO) trial[60] confirms that there is a linear relationship between hyperglycaemia and birth weight, even at glucose levels not usually considered abnormal during pregnancy. The Australian Carbohydrate Intolerance Study in Pregnant Women (ACHOIS)[58] confirms that treating hyperglycaemia can substantially reduce both the incidence of large for gestational age babies and other perinatal complications. Thus, metformin, by reducing insulin resistance, could have a major impact in reducing excess birth weight in obese pregnant women.

This study is timely because of emerging evidence about the safety and efficacy of metformin. It is a licensed first line therapy for the treatment of type 2 diabetes and is endorsed by the National Institute for Health and Care Excellence (NICE) as a treatment for gestational diabetes[99].

There are few previous studies testing the effects of metformin in pregnant women in the absence of diabetes. A small randomised study of 40 pregnant women with a history of polycystic ovary syndrome (PCOS), a condition often accompanied by insulin resistance, showed that metformin reduced pre-defined pregnancy complications from 32% in the placebo group (n=22) to 0% in the metformin group (n=18) (p=0.01)[82]. However, a larger study by the same group, which randomised 274 women with PCOS to either metformin or placebo throughout pregnancy, showed no significant differences in the prevalence of preterm birth, pre-eclampsia and gestational diabetes[83]. There was also no difference in birth weights of the babies in the two groups. However, the mean BMI of the participants in these studies was less than 30 kg/m^2. In the Metformin in Gestational diabetes (MiG) study, which compared the effect of metformin versus insulin in women with gestational diabetes [100], there were no differences in birth weight between the offspring. However, weight gain in pregnancy, a known additional driver of birth weight[90], was lower in the metformin group (difference of 1.6 kg, p<0.001). By two years of age, offspring of

the participants randomised to metformin had greater subcutaneous fat, but overall body fat was the same as in children whose mothers were treated with insulin alone, suggesting that the 'metformin offspring' have smaller deposits of visceral, metabolically active fat[101]. Further follow-up is required to examine whether these findings persist into later life and whether children exposed to metformin will be more insulin sensitive.

Importantly, if metformin is to be effective in reducing high birth weight in obese women, it should not increase the proportion of babies with low birth weight. The studies mentioned above, and another small study, on women with a mean BMI less than 30 kg/m^2 are reassuring on this point[82, 83, 102].

2.3. Hypothesis

We hypothesise that metformin administered to obese women during pregnancy will reduce birth weight centile in their babies and consequently their future life risk of obesity and metabolic syndrome.

2.4. Aim

To determine if metformin administered to obese women during pregnancy reduces birth weight centile in their babies, using birth weight as a surrogate marker of the future life risk of obesity and metabolic syndrome in the offspring.

2.5. Objectives

2.5.1. Primary objective

To determine the efficacy of metformin (up to 2500 mg daily), given to obese pregnant women from between 12 and 16 weeks gestation until delivery, in reducing gestational age-adjusted and sex-adjusted birth weight centile of the baby.

2.5.2. Secondary objectives

- To determine the pattern of association between insulin resistance and adverse pregnancy outcomes, including incidence of pregnancy-induced hypertension, pre-eclampsia, caesarean section, postpartum haemorrhage, maternal weight gain during pregnancy, and incidence of the baby's admission to the neonatal unit.
- To determine the effect of metformin on maternal body composition
- To determine the effect of metformin on neonatal body composition
- To determine the effect of metformin on maternal inflammatory and metabolic variables (measured at 28 and 36 weeks' gestation) and on neonatal inflammatory variables (measured in cord blood at birth).
- To confirm that metformin does not increase the rate of babies with a low birth weight centile.
- To determine the efficacy (as opposed to the effectiveness) of metformin when analysis is restricted to those with pharmacological circulating levels of drug.

A series of nested sub-studies will also be performed with the following objectives

- To determine the effect of metformin on maternal cortisol levels in obese pregnant women
- To determine the effect of metformin on hepatic and peripheral insulin sensitivity at 36 weeks' gestation in obese pregnant women
- To determine the effect of metformin on endothelium-dependent flow-mediated dilatation in obese pregnant women
- To determine the effect of metformin on maternal subcutaneous and visceral adipose tissue deposition and hepatic and skeletal muscle ectopic fat deposition during pregnancy
- To determine the effect of metformin on fetal liver volume and subcutaneous fat deposition

- To determine the effect of metformin on myometrial contractility and myometrial glycogen storage in obese pregnant women
- To determine the effect of metformin on placental glucocorticoid receptor and 11βHSD 1 and 2 mRNA levels.

2.6. Centre(s)

Seventeen hospitals in the UK.

2.7. Design

The design is a double-masked randomised placebo-controlled trial, with embedded sub-studies to explore mechanism of action of metformin, in a population of ~400 obese pregnant women (Figure 1).
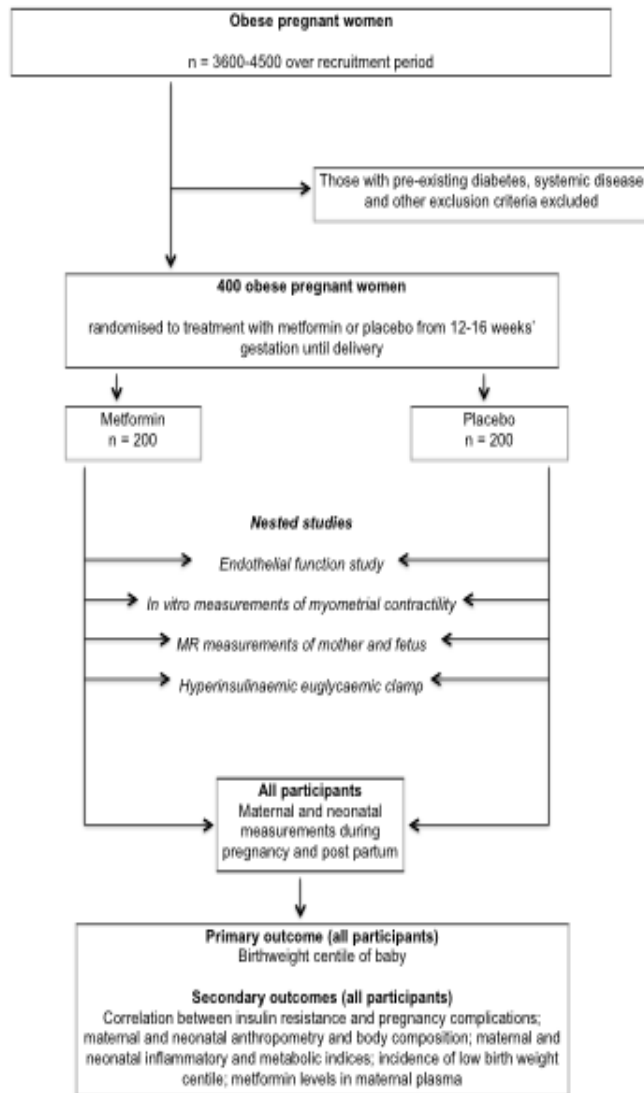
Figure 1 Flow chart of participants in the EMPOWaR trial

2.8. Inclusion and exclusion criteria

2.8.1. Screening phase inclusion criteria

- Caucasian obese (BMI ≥ 30 kg/m^2) pregnant women between 12^{+0} and 16^{+0} weeks' gestation;
- Age greater than or equal to 16 years;
- Signed informed consent form.

2.8.2. Screening phase exclusion criteria

- Non-Caucasian;
- BMI < 30 kg/m^2;
- Gestation $> 16^{+0}$ weeks;
- Pre-existing diabetes;
- Gestational diabetes in a previous pregnancy;
- Systemic disease at the time of trial entry, with the disease either requiring regular medication or having required treatment with systemic steroids in the past 3 months;
- Previous delivery of a baby $< 3^{rd}$ centile by weight;
- Previous pregnancy complicated by pre-eclampsia prompting delivery before 32 weeks' gestation;
- Known sensitivity to metformin hydrochloride or any of the excipients;
- Acute conditions at the time of trial entry with the potential to alter renal function such as dehydration sufficient to require intravenous infusion, severe infection, shock, intravascular administration of iodinated contrast agents;
- Acute or chronic diseases which may cause tissue hypoxia such as cardiac or respiratory failure, recent myocardial infarction, hepatic insufficiency, acute alcohol intoxication, alcoholism;
- Lactation;
- Multiple pregnancy.

2.8.3. Randomisation exclusion criteria following screening

- Gestational diabetes mellitus (GDM) in index pregnancy (diagnosed with 75 g oral glucose tolerance test using WHO criteria, see Table 1). Participants are also excluded if glucose tolerance test is diagnostic of GDM based on the criteria used in the recruiting centre.

- Known liver or renal failure or dysfunction at the time of trial entry tested prior to randomisation (defined as variable outwith the reference range shown in Table 2).

Time (hours)	Blood glucose (mmol/L)
0	≥ 7.0
2	≥ 7.8

Table 1 WHO diagnostic criteria for gestational diabetes following 75 g oral glucose tolerance test

Variable	Range
Urea	2.5-6.6 mmol/L
Creatinine	< 85 μmol/L
Sodium	135-145 mmol/L
Potassium	3.6-5.0 mmol/L
Bilirubin	3-16 μmol/L
ALT	10-60 IU/l

Table 2 Reference ranges for biochemical parameters used in the EMPOWaR protocol

2.8.4. Ineligible and non-recruited participants

No further information will be collected on women who are ineligible solely because of abnormalities in glucose tolerance, liver or renal function, other than the number of such women for inclusion in trial metrics.

Telephone or face-to-face interviews will be carried out to explore the reasons for eligible women declining to participate.

2.9. Methods

We will recruit women attending maternity hospitals in the UK. Women who fulfil all the potential eligibility criteria and who express an interest in the study will be provided with the participant information sheet and given at least 24 hours to consider participation. They will then be asked to provide written informed consent. Following consent, participants will have screening blood tests including liver and renal function and a 75 g oral glucose tolerance test, to determine eligibility for randomisation. Participants will have demographics, medical history, height, weight and anthropometry measurements (waist, hips, mid-arm, mid-thigh circumference; tricep, bicep and subscapular skinfolds) recorded at this visit.

2.9.1. Randomisation

Eligible subjects will be randomly assigned to active treatment with metformin or an identical looking placebo. This will be documented in the patient's paper case record and/or computer file to show the woman's participation in the trial.

Participants will be randomised via a web portal connected to a central randomisation facility based at the trial data centre, the Edinburgh Clinical Trials

Unit, University of Edinburgh. Baseline eligibility data will be required before randomisation. Participants will be randomised in a 1:1 ratio to metformin or placebo. Randomisation will be stratified by treatment centre and BMI 30-39 kg/m^2 versus >40 kg/m^2.

2.9.2. Concealment of allocation

Randomising subjects to active or placebo tablets will achieve concealment of allocation. Placebo tablets will appear identical to active treatment, so that the participant is masked to treatment allocation. The outcomes will be measured by clinicians and investigators masked to treatment allocation. Masking will not be broken until after data entry is complete, the validity of the data is checked, all queries resolved, the patient populations agreed and the database locked. Any clinically indicated emergency unmasking will be recorded prospectively.

2.9.3. Intervention

Metformin tablets (or matched placebo) 500 mg administered from as soon as practicable after the point of randomisation (and certainly between 12 and 16 weeks' gestation) until delivery of the baby. The dose regimen is as follows: week 1, 500 mg once daily; week 2, 500 mg twice a day; week 3, 500 mg three times a day; week 4, 500 mg morning and lunchtime and 1000 mg in the evening; week five until delivery of the baby, 1000 mg in the morning and evening and 500 mg at lunchtime. All doses are taken with food and dose escalation should continue to either the maximum tolerable dose or 2500 mg, whichever is higher.

2.9.4. Dose changes

The local investigator or participant may alter the treatment regimen at his/her discretion, so long as the maximum daily dose does not exceed 2500 mg. Changes to the treatment dose will be recorded in the electronic case report form (eCRF) as soon as practicable.

2.9.5. Other medications

Alcohol is prohibited due the increased risk of lactic acidosis. Iodinated contrast agents may increase the risk of renal failure, hence, if they are required, metformin should be discontinued for at least 48 hours from immediately prior to contrast agent administration until after renal function has been re-evaluated and found to be normal. Clinicians prescribing glucocorticoids (systemic and local routes), β2-adrenoreceptor agonists and ACE inhibitors should be aware that they might amplify or diminish the hypoglycaemic effect of metformin.

2.9.6. Study assessments

Following randomisation, study assessments for glucose tolerance testing and other tests will occur as detailed in Table 4 and Table 4.

2.9.6.1. Gestation	10-16 weeks Screening	10-16 weeks Consent	12-16 weeks Randomisation	18-20 weeks Study visit (could be by telephone)	28 Weeks Study visit	36 Weeks Study vsiit	Term Study visit (could be by telephone)	Delivery Study vsiit	3 months postnatally Study visit
Review inclusion and exclusion criteria, give patient information leaflet	X								
Consent Form		X							
Demographics and medical history		X							
Height, weight and anthropometry		X				X			X
Bloods for liver function/renal function/full lipid profile/CRP		X				X			
75g OGTT and stored sample for inflammatory and metabolic indices		X			X	X			
Randomisation			X						
Study Drug dispensed			X		X				
Unused Study Drug /packaging returned					X			X	
Review SAEs, complete side effect questionnaire, record complications				X	X	X	X	X	
Labour and delivery information including birthweight, mode of delivery, EBL								X	
Baby's weight and anthropometry								X	X

Table 3 Summary of EMPOWaR study visits for all participants

Visit Number	1	2	3	4	5	6	7	8	9
Gestation	10-16 weeks	10-16 weeks	12-16 weeks	18-20 weeks	28 Weeks	36 Weeks	Term	Labour	3 months postnatally
Saliva samples for cortisol measurements			X		X	X			
Bodpod measurements		X (OR VISIT 3)	X (OR VISIT 2)			X			X
Hyperinsulinaemic euglycaemic clamp						X			
FMD			X			X			
MR scan					X	X			
Cord blood & placenta biopsy								X	
Myometrium biopsy (if delivered by CS)								X	
Peapod measurements								X	X

Table 4 Summary of EMPOWaR study visits for participants enrolled in substudies

Maternal anthropometric measurements recorded at baseline, 36 weeks' gestation and 3 months postpartum, will include waist; hip; upper arm and thigh circumference and bicep, tricep and subscapular skinfold thickness. Neonatal anthropometric measurements recorded within 72 hours of birth and at 3 months of age will include head circumference, and tricep and subscapular skinfold thickness. Length and weight will be recorded in order to calculate ponderal index.

Fasting maternal blood samples obtained at baseline, 28 and 36 weeks' gestation will be stored for future analysis of inflammatory and metabolic indices, including (but not limited to) insulin, C reactive protein (CRP), interleukin (IL)-6, leptin, plasminogen activator inhibitor (PAI)1/PAI2 ratio, cortisol, lipids, and non-esterified fatty acids. Cord blood obtained at the time of delivery will also be taken for measurement of glucose and stored for future measurement of the inflammatory and metabolic indices listed previously.

Women who develop gestational diabetes (according to site specific diagnostic criteria) should be given insulin whilst maintaining study treatment and blinding.

Table 4 documents the timing of the nested sub-studies in which a subgroup of subjects will participate. A summary of these is given below.

2.9.7. Saliva samples

Diurnal cortisol levels will be measured in saliva samples collected at baseline, 28 and 36 weeks' gestation. Saliva will be collected in salivettes at bed-time and on waking. Samples will be stored at -80 °C. Cortisol will be measured by enzyme-linked immunosorbant assay (ELISA). Participants at all study sites will be invited to provide saliva samples.

2.9.8. Body composition

Maternal fat mass will be measured using air displacement plethysmography (BOD POD®, www.lifemeasurement.com) at baseline, 36 weeks' gestation and 3 months postpartum. Neonatal fat mass will be measured using the same technique (PEA POD®, www.lifemeasurement.com) within 72 hours of birth and at 3 months of age. Only participants at the Edinburgh site will be invited to take part in this substudy.

2.9.9. Hyperinsulinaemic euglycaemic clamp

A subgroup of women will undergo a hyperinsulinaemic euglycaemic clamp study at 36 weeks' gestation to characterise the relative effects of metformin on hepatic and peripheral insulin sensitivity. Only participants at the Edinburgh site will be invited to take part in this substudy.

2.9.10. Flow mediated dilatation

Endothelium-dependent flow-mediated dilatation will be measured in a subgroup of participants at baseline and again at 36 weeks' gestation. Change in diameter of the brachial artery following a flow stimulus created by arterial occlusion will be measured using continuous two-dimensional grayscale ultrasound imaging. Only participants at the Edinburgh site will be invited to take part in this substudy.

2.9.11. MRI and spectroscopy

A subgroup of participants will be scanned at 28 and 36 weeks' gestation using a Verio 3 Tesla MRI system. T1-weighted acquisitions will be used to measure maternal subcutaneous and visceral fat; fetal liver volume; and fetal subcutaneous fat. Images will be analysed using the software programme SliceOmatic. This programme uses knowledge-based image processing to label pixels as fat or non-fat components and the adipose tissue mass derived using a mathematical model. Hepatic and skeletal muscle lipid content will be measured using ^{1}H-magnetic

resonance spectroscopy. Only participants at the Edinburgh site will be invited to take part in this substudy.

2.9.12. Myometrial biopsies

A biopsy of the lower segment myometrium will be obtained from consenting subjects who are delivered by caesarean section. The biopsies will be divided, with one portion placed in physiological saline for contractility studies and the other snap frozen for glycogen storage measurements. Participants at all study sites will be invited to consent to myometrial biopsy in the event of delivery by caesarean section.

2.9.13. Participant compliance

Compliance will be recorded in a treatment diary and by counting of unused tablets. Additionally, gas chromatography mass spectrometry will be used to analyse metformin levels in blood samples from participants in the third trimester.

2.10. Outcomes

2.10.1. Primary outcome

Z-score corresponding to the gestational age-adjusted and sex-adjusted birth weight centiles of the baby.

2.10.2. Secondary outcomes

- Maternal insulin resistance at 36 weeks' gestation, which will be correlated with adverse pregnancy outcomes.
- Maternal anthropometry and body composition at 16 and 36 weeks' gestation and 3 months postpartum.
- Baby anthropometry and body composition at birth and 3 months of age.

- Maternal inflammatory markers, lipid and fatty acid indices prior to commencing treatment and again at 28 and 36 weeks' gestation including CRP, IL-6, leptin, lipid profile, non-esterified fatty acids, polyunsaturated fatty acids and PAI1/PAI2 ratio.

- Neonatal CRP, glucose, insulin and other inflammatory and metabolic indices as previously described (measured in cord blood at birth).

- Incidence of low birth weight centile.

- Gas chromatography mass spectrometry measurement of metformin in maternal plasma to determine adherence.

Secondary outcomes from nested sub-studies;

- Maternal salivary cortisol levels at baseline, 28 and 36 weeks' gestation.

- Hepatic and peripheral insulin sensitivity at 36 weeks' gestation as measured by the hyperinsulinaemic euglycaemic clamp technique.

- Maternal brachial artery endothelium flow-mediated dilatation measured at 16 and 36 weeks' gestation.

- Maternal subcutaneous and visceral adipose tissue deposition and hepatic and skeletal muscle ectopic fat deposition assessed using MRI and magnetic resonance spectroscopy.

- Fetal liver volume and fetal subcutaneous fat deposition assessed using MRI.

- In vivo measurements of myometrial contractility on myometrial biopsies obtained at the time of caesarean section.

- Placental glucocorticoid receptor and $11\beta HSD$ 1 and 2 mRNA levels.

2.11. Side effects and adverse events reporting

Participants are instructed to contact their investigator at any time after consenting to randomisation if any symptoms develop. In the case of any events, the investigator will initiate the appropriate treatment according to their medical judgement. Participants with adverse events (AEs) present at their last visit will be followed up until resolution of the event. All AEs and serious adverse events (SAEs) that occur

after randomisation will be recorded in detail in the participant's medical notes. SAEs occurring in the mother or baby from the time a participant is randomised until 30 days after stopping taking study treatment or until 28 days after delivery (whichever is later) will be reported to the co-sponsors using the trial documentation. The standard definition of a serious adverse event will be used.[103] For the purposes of this study the following events will not be considered SAEs: miscarriage; preterm labour; preterm prelabour spontaneous rupture of membranes; preterm delivery in the maternal interest; preterm delivery in the fetal interest; hospitalisation for pregnancy induced hypertension; hospitalisation for maternal discomfort; hospitalisation for rest; hospitalisation for observation or monitoring for which the woman is admitted for a period of less than 12 hours; delivery complications such as caesarean section or post partum haemorrhage; admission of the baby to the neonatal unit for a period of up to 14 days.

2.12. Statistical analysis plan, including sample size and power calculations

2.12.1. Birth weight centiles

In a previous study, the mean (SD) birth weight in a cohort of obese women (mean BMI 34 kg/m^2) was 4.0 (0.6) kg.[104] We hypothesise that metformin will reduce mean birth weight by 0.2 kg, corresponding to a reduction in birth weight centile of 0.33SD. We believe that this reduction is clinically relevant, but is a relatively conservative estimate of likely reduction in birth weight centile induced by metformin, given that mean birth weight in the study described above in a parallel non-obese cohort was 3.4 kg. A sample size of 143 in each group will have 80% power to detect a difference in mean birth weight centile of 0.33 SD (the difference between a placebo mean of 4.0 kg and a metformin mean of 3.8 kg) at the 5% significance level (2-sided) using a two group t-test; a sample size of 163 in each group will give the study 85% power to detect these differences. In practice we will recruit a larger sample size to allow loss to follow-up.

2.12.2. Type of analysis and statistical tests

The primary analysis will be by intention to treat. Mean birth weight centile will be compared between the two groups using a two-sample t-test, but with the analysis stratified for the same factors as the randomisation. Correlations within the metformin and placebo groups will be used to determine associations between insulin resistance and adverse pregnancy outcomes.

2.12.3. Interim analysis and stopping rules

No formal interim analysis is planned (other than those requested by the Data monitoring Committee (DMC)).

2.12.4. Committee oversights

There is an independent Trial Steering Committee and an independent Data Monitoring Committee to oversee the safety of the participants in the trial.

2.13. Discussion

Despite recognition that obesity represents a major public health problem, and that adult obesity may have its origins before birth, there is a lack of any effective intervention for obese pregnant women to improve pregnancy outcomes and reduce the future life risk of obesity for their offspring. In light of convincing evidence that the harmful effects of obesity during pregnancy are related to hyperglycaemia or insulin resistance, treatment during pregnancy with metformin is an exciting potential therapy. The Efficacy of Metformin in Pregnant Obese Women, a Randomised controlled (EMPOWaR) trial is designed to establish whether improving insulin sensitivity in pregnancy mitigates some of the adverse risk associated with obesity, with the primary aim of examining the effect on the birth weight of the baby, using birth weight as a surrogate marker of future life risk of

obesity. A series of embedded mechanistic studies will help us to understand more about the mechanism of effect of metformin.

If our study finds metformin to be beneficial in reducing excess birth weight in obese pregnant women, it presents a potential future therapy where none currently exist. Clearly further large studies will be required to corroborate our findings. To our knowledge there is currently only one other randomised double-masked placebo-controlled trial in progress which aims to recruit 850 women with a BMI >35 kg/m2 and is due to complete in September 2014 (Shehata, http://clinicaltrials.gov/show/NCT01273584).

As for any drug trial in pregnant women, safety is clearly a priority. Metformin has been used for decades in pregnant women with no evidence of any teratogenic effects. We do not expect any adverse effects from teratogenicity in our study population. There is no evidence from previous studies that metformin increases the incidence of babies with a low birth weight centile and we hope to confirm this with our data. Longer-term follow-up studies of the offspring will be needed to assess the benefits or adverse effects in later life of antenatal exposure to metformin.

Finally, metformin is an inexpensive drug, costing under £5 per month. Obesity and the associated maternal and fetal complications are a huge financial burden on health services[105]. If metformin were found to be effective, its use could contribute to significant financial savings for health services.

The subsequent chapters of this thesis will describe the findings of this trial as described. The results of the primary outcome and the main secondary outcomes are reported in Chapter 3. The results of the mechanistic substudies performed by the candidate (the HEC study, the MRI studies and the FMD study) are described in

Chapters 4, 5 and 6. A full report of all the methods and results, including the other substudies, described in this protocol is provided in the NIHR report, included as an appendix to this thesis.

3. Chapter 3   Effect of metformin on maternal and fetal outcomes in obese pregnant women (EMPOWaR): a randomised, double-blind, placebo-controlled trial

The work in this chapter has been published in the Lancet Diabetes and Endocrinology

Chiswick C, Reynolds RM, Denison FC, *et al*. Effect of metformin on maternal and fetal outcomes in obese pregnant women (EMPOWaR): a randomised, double-blind, placebo-controlled trial. *Lancet Diabetes Endocrinol* 2015;**3**:778-86

3.1. Background

The adverse effects of maternal obesity on short-term pregnancy complications include pre-eclampsia[9], caesarean section, longer maternal and neonatal hospital stay, maternal haemorrhage, infant mortality[106] and stillbirth[94]. Maternal obesity during pregnancy is also associated with higher birthweight and neonatal fat mass[94, 107].

Accumulating data suggest that maternal obesity might predispose offspring to later life obesity, with high birthweight being a marker for increased risk. Correlations between high birthweight and adult obesity have been reported in large epidemiological studies[15, 16], a systematic review[17] and a validated prediction model[108]. The rapid rise in the prevalence of both high birthweight[36] and maternal obesity mean that their links with later life obesity are a major concern. Indeed, in a recent record linkage study[18], we showed that maternal obesity was associated with a 35% increase in the hazard of all cause offspring mortality in adulthood, even after adjustment for confounders. As such, an effective intervention applied during pregnancy could have a major effect on interruption of the cycle of maternal obesity

and offspring obesity and ill health, thus helping to reverse the upward secular trend in obesity prevalence.

Much evidence implicates insulin resistance (i.e. when a defined concentration of insulin does not effect a predictable metabolic response) and hyperglycaemia as the mechanism by which maternal obesity causes excessive neonatal birthweight. Obese pregnant women are significantly more insulin resistant and hyperglycaemic than are pregnant women of a normal weight[62] and several large studies including the Camden study[109] and the HAPO study[60] show a positive association between high glucose concentrations and macrosomia, even at glucose concentrations regarded as normal during pregnancy. Additionally, a Cochrane review protocol[110] has outlined additional potential benefits on mother and baby of metformin in obese pregnant women.

In view of these findings, we did this EMPOWaR study[111] to test the hypothesis that the insulin sensitising drug metformin would reduce birthweight when given to obese women during pregnancy. On the basis of findings form other epidemiological studies[15, 16, 84] a reduction in birthweight would be expected to result in a reduction in future life risk of obesity and metabolic syndrome in the offspring.

3.2. Methods

3.2.1. Study design and participants

We did this randomised, double-blind, placebo-controlled trial in antenatal clinics at 15 National Health Service (NHS) hospitals in the UK. Eligible women were aged 16 years or older, had a BMI of 30 kg/m. or more, and were between 12 and 16 weeks' gestation. We excluded non-white women and those with: pre-existing diabetes; gestational diabetes in a previous pregnancy; gestational diabetes diagnosed in the index pregnancy before randomisation; systemic disease at the time of trial entry (requiring either regular drugs or treatment with systemic corticosteroids in the past 3 months); previous delivery of a baby smaller than the 3rd percentile for weight; previous pregnancy with pre-eclampsia prompting delivery before 32 weeks' gestation; known hypersensitivity to metformin hydrochloride or any of the excipients; known liver failure; known renal failure; acute disorders at the time of trial entry with the potential to change renal function, such as dehydration sufficient to require intravenous infusion, severe infection, shock, intravascular administration of iodinated contrast agents, or acute or chronic diseases that might cause tissue hypoxia (e.g. cardiac or respiratory failure, recent myocardial infarction, hepatic insufficiency, acute alcohol intoxication, or alcoholism); lactating women; and women with multiple pregnancy.

The study was approved by the Scotland A research ethics committee (reference number 10/MRE00/12) and the Medicines and Healthcare products Regulatory Agency (EudraCT number 2009-017134-47). All participants provided written information consent. The protocol has been published elsewhere[111] and is available online.

3.2.2. Randomisation and masking

We randomly assigned participants (1:1), via a web-based computer-generated block randomisation procedure (block size of two to four), to receive metformin or

placebo. Randomisation was stratified by study site and BMI band (30–39 vs ≥40 kg/m.). Participants, caregivers, and study personnel were masked to treatment assignment. Members of the independent Data Monitoring Committee had access to unmasked data reports, but had no contact with study participants.

3.2.3. Procedures

Demographics, medical history, and maternal anthropometry were recorded at baseline. A formal 75 g oral glucose tolerance test was done in addition to screening for liver and renal function. We excluded participants with impaired renal function (urea >6.6 mmol/L, creatinine >85 μmol/L, sodium >145 mmol/L, potassium >5.0 mmol/L), or liver function (bilirubin >16 μmol/L, alanine transferase >60 IU/l), or with abnormal lactate (according to local laboratory reference range) or gestational diabetes defined by WHO criteria (fasting glucose ≥7.0 mmol/L and 2 h glucose ≥7.8 mmol/L), or any other local hospital criteria (e.g. International Association of Diabetes and Pregnancy Study Groups [IADPSG][112]).

Participants received oral metformin 500 mg or matched placebo tablets, in a dose of up to five tablets daily in two to three divided doses. Treatment was initiated at 12–16 weeks' gestation and continued until delivery of the baby. Treatment started at one 500 mg tablet once a day at week 1, and escalated by one tablet a day each week over 5 weeks, to reach either the maximum tolerable dose or the maximum permitted dose of 2500 mg, whichever was lower. In the case of side effects, participants were advised to reduce the current dose to that of the previous week, and wait for 1 week before increasing the dose again. The local investigator was allowed to change the treatment regimen at their discretion, as long as the maximum daily dose did not exceed 2500 mg in three divided doses. Participants were asked to keep a diary of drug intake and to bring all drugs to each study visit to monitor compliance. Randomised participants were reviewed face to face or by telephone at 18–20, 28, 36, and 40 weeks' gestation; around the time of delivery; and 3 months postnatally. Pregnancy complications were recorded and women were asked to complete a side-

effect questionnaire at each review visit until delivery. Maternal anthropometry was repeated at 36 weeks' gestation and 3 months postnatally. The glucose tolerance test was repeated at 28 and 36 weeks' gestation, and blood was stored for measurement of inflammatory and metabolic indices. The protocol recommended that women who developed gestational diabetes should be given insulin whilst maintaining study treatment and blinding. The baby's weight and anthropometry were recorded at delivery and at the 3-month postnatal visit.

3.2.4. Outcomes

The primary outcome was Z score corresponding to the gestational age, parity, and sex-standardised birthweight percentile of liveborn babies delivered at 24 or more weeks' gestation. The main secondary outcome was maternal insulin resistance at 36 weeks' gestation. Other secondary outcomes included maternal fasting glucose and insulin and 2 h glucose at 36 weeks; maternal anthropometry and body composition; baby anthropometry and body composition; maternal inflammatory and metabolic outcomes at 36 weeks, including C-reactive protein (CRP), cholesterol, HDL, LDL, triglycerides, interleukin (IL)-6, leptin, serum cortisol, non-esterified fatty acids, and the ratio of plasminogen activator inhibitor 1 to 2; incidence of low birthweight percentile (<3rd and <10th); incidence of other adverse maternal and neonatal outcomes, including maternal symptoms; maternal plasma metformin concentration to explore tablet taking in the metformin group; and the maternal metabolic (fasting glucose and insulin and 2 h glucose) and inflammatory markers at 28 weeks. The methods for detection of the blood analytes have been described elsewhere.[111] Secondary mechanistic outcomes as outlined in the published protocol[111] were obtained in a subset of participants and will be reported elsewhere. We made some changes to the protocol after recruitment began, but before generation of the statistical analysis plan, publication of the protocol[111], and unmasking and analysis. Specifically, maternal insulin resistance at 36 weeks' gestation was originally a co-primary outcome, but was relegated to a secondary outcome when a substantial proportion of participants did not provide a blood sample at 36 weeks. Additionally,

we used patient self-reporting of tablet taking to establish treatment compliance and inform the per-protocol analysis.

3.2.5. Statistical analysis

We calculated that a sample size of 143 women in each group would provide 80% power, and a sample size of 163 women in each group would provide 85% power, to detect a difference in mean birthweight percentile of SD 0.33 (equivalent to the difference between a placebo mean of 4.0 kg and a metformin mean of 3.8 kg) at a two-sided 5% significance level with a two-group t test. We initially aimed to randomise 400 women based on anticipated high compliance and follow-up rates, but in a protocol amendment increased our sample size to 450 women when anecdotal evidence (without formal testing) suggested that compliance was lower than anticipated. We did our primary analysis in the modified intention-to-treat population. We also did per-protocol analyses, in which we compared outcomes amongst participants who were compliant with treatment. Compliance was determined before review of the data or unmasking. To measure compliance we calculated the number of weeks from randomisation to delivery for each woman; participants reporting (via their study diary) that they took at least one tablet on at least 4 days per week for at least half of those weeks were deemed to have been compliant. We did not use plasma metformin to measure compliance as no such measure of compliance could be done for placebo. We did exploratory analyses of secondary outcomes. No formal adjustment was made to any p values to allow for the large number of secondary endpoints analysed, and thus p values for secondary analyses need to be interpreted conservatively. We also did post-hoc analyses of safety outcomes of all reported serious adverse events and the combined adverse outcome of stillbirth, neonatal death, termination of pregnancy, or miscarriage. We derived birthweight percentiles and Z scores of birthweight percentiles (livebirths only) for each patient after adjustment for sex, gestational age, and parity (nulliparous vs multiparous) with population-derived charts[113]. We used a linear regression model adjusted for treatment centre and BMI band (30–39 vs ≥40 kg/m.) to compare Z scores between the groups and to obtain the adjusted mean difference

with 95% CI. This method was also used for other continuous outcomes including glucose and insulin and homeostatic model assessment of insulin resistance (HOMA-IR). When necessary, we did log transformations to achieve normal distribution of data before statistical testing. For assessment of CRP concentration in the umbilical cord, we used Kruskal–Wallis one-way analysis of variance because this variable could not be transformed into a normal distribution. We used unadjusted logistic regression for binary outcomes and Fisher's exact test when the event counts were small. Relevant denominators were either all participants randomised for whom information was available, or those having a livebirth for whom information was available. We did analyses with SAS (version number 9.3). A trial steering and a data and safety monitoring committee oversaw the study. The trial was registered, ISRCTN number 51279843.

3.2.6. Role of the funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

3.3. Results

Between Feb 3, 2011, and Jan 16, 2014, inclusive, we randomly assigned 449 participants to the placebo group (n=223) or the metformin group (n=226), of whom 434 (97%) were included in the modified intention-to-treat analysis (Figure 2).
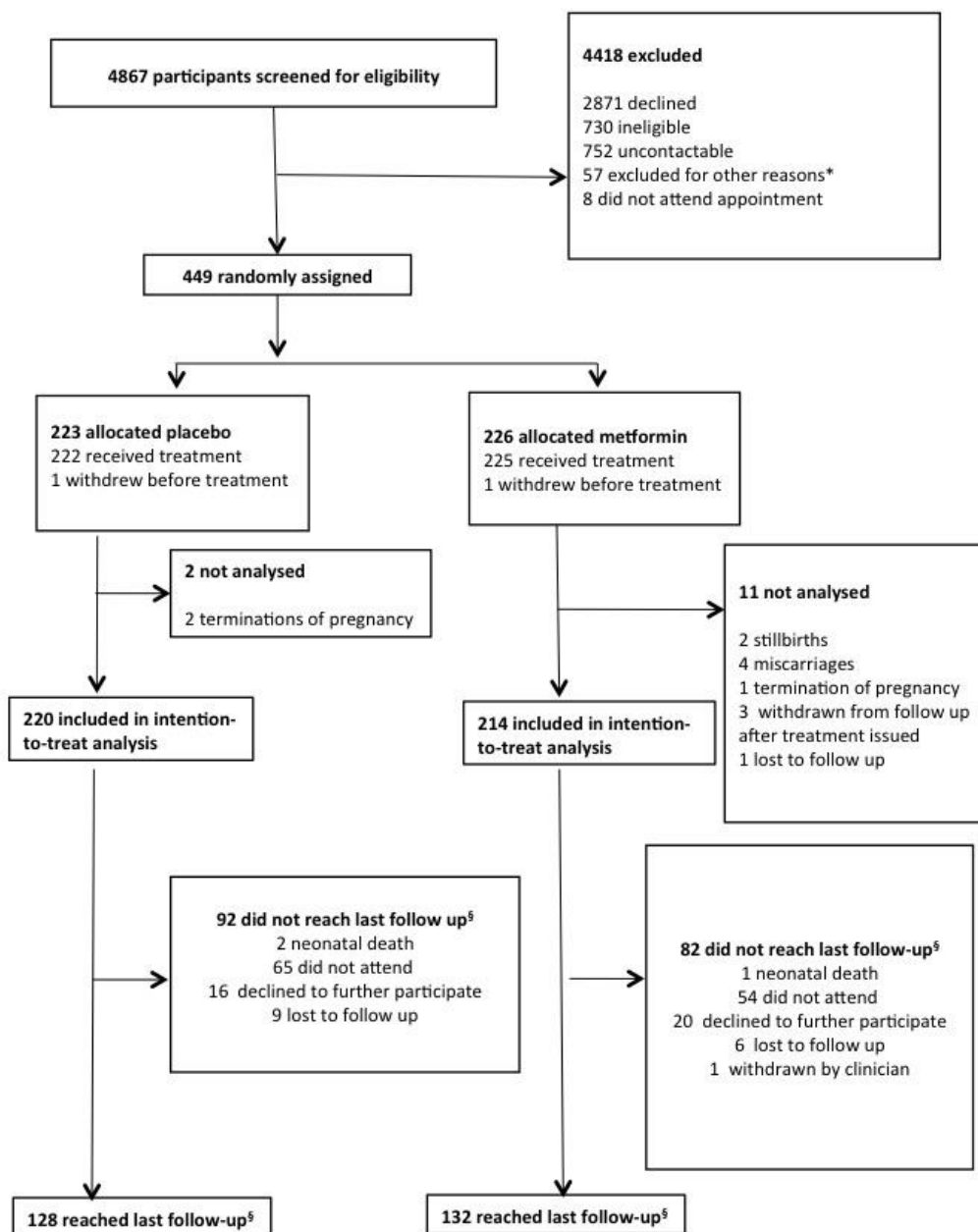
Figure 2 EMPOWaR trial profile

The most common reasons for non-participation were a concern that study drugs might be harmful to the baby, and low awareness about the adverse effects of obesity

on pregnancy outcome. Baseline demographics, medical history, and maternal anthropometry were similar between groups (Table 5).

	Placebo		Metformin	
	n=223		n=226	
Demographics and lifestyle (participant)	**Mean or number (n)[1]**	**SD or %**	**Mean or number (n)**	**SD or %**
Age (years)	28.9	5.1	28.7	5.8
Currently smokes	31	13.9%	40	17.7%
Currently drinks alcohol	9	4.0%	3	1.3%
Illicit drug use	1	0.4%	0	0%
Highest educational qualification				
school for $\leq$ 16 years	79	35.4%	75	33.1%
school for $\geq$ 16 years	144	64.6%	151	66.9%
At least one previous pregnancy ≥12 weeks' gestation	161 (220)	73.2%	147	65.0%
Systolic blood pressure (mmHg)	119.4	10.4	117.6	10.8
Diastolic blood pressure (mmHg)	68.9	7.3	68.0	7.8

---

[1] For this table "n" is shown where it is different from the number at the top of the table

Gestation at baseline (days)	98.9	8.7	99.1	8.1

Medical history (participant)				
Pre-eclampsia or pregnancy induced hypertension	7	3.1%	10	4.4%
Pre- pregnancy hypertension requiring treatment	2	0.9%	1	0.4%
Polycystic ovary syndrome	21	9.4%	28	12.4%
Depression requiring treatment	71	31.8%	48	21.2%
Anxiety requiring treatment	20	9.0%	15	6.6%
Family history				
Cardiovascular disease	69	30.9%	71	31.4%
Pre-eclampsia	22	9.9%	19	8.4%
Diabetes	101	45.3%	99	43.8%
Other	96	43.0%	109	48.2%

Anthropometry	

Height (cm)	165.1	5.9	165.5	5.9
Weight (kg)	102.9	17.0	103.6	15.5
BMI calculated (kg/m^2)	37.7	5.6	37.8	4.9
Waist (cm)	108.7 (222)	13.5	110.1 (225)	11.9
Hip (cm)	126.4 (222)	12.1	127.4 (225)	11.8
Mid arm (cm)	36.3 (220)	5.0	36.7 (221)	4.7
Mid-thigh (cm)	64.1 (219)	7.7	64.2 (222)	6.9
Tricep skinfold (mm)	31.2 (222)	9.7	31.9 (222)	10.8
Bicep skinfold (mm)	25.7 (222)	10.0	27.4 (222)	10.9
Subscap skinfold (mm)	32.0 (222)	12.2	32.6 (220)	11.8
Maternal % fat[2]	46.8 (48)	5.6	48.2 (53)	5.2

Blood tests recruitment visit (participant)				
Fasting glucose (mmol/L)	4.39	0.34	4.41	0.40

---

[2] Measured only in Edinburgh participants

2 h glucose (mmol/L)[3]	5.50	1.09	5.20	1.08
Fasting insulin (µIU/ml)	22.08 (189)	10.20	21.95 (188)	12.26
HOMA-IR score[4]	4.36 (189)	2.16	4.36 (188)	2.76
CRP (mg/L)	11.1 (221)	7.4	10.7 (223)	6.9
Cholesterol (mmol/L)	4.87 (216)	1.15	4.88 (214)	1.09
HDL (mmol/L)	1.67 (215)	0.39	1.64 (214)	0.38
LDL (mmol/L)	2.91 (194)	0.78	2.89 (191)	0.86
Triglycerides (mmol/L)	1.51 (216)	0.53	1.43 (214)	0.56
IL-6 (mmol/L)	2.77 (189)	5.50	2.63 (188)	4.37
Leptin (ng/ml)	93.6 (189)	42.1	98.5 (188)	40.3
Serum cortisol (nmol/L)	396.4 (189)	143.6	431.0 (188)	178.8
NEFA (mmol/L)	0.52 (189)	0.20	0.48 (188)	0.18
PAI-1 to PAI-2 ratio	1.48 (131)	1.39	1.77 (128)	5.22

---

[3] After a 75g oral glucose challenge
[4] Fasting glucose (in mmol/l) x insulin (in µIU/ml)/22.5

Putative father details				
Height (cm)	178.5 (204)	8.3	177.1 (202)	13.7
Weight (kg)	92.3 (187)	22.5	93.5 (188)	25.8
Ethnic origin				
White	214	96.0%	210 (224)	93.8%
Mixed	4	1.8%	4 (224)	1.8%
Asian	0	0%	3 (224)	1.3%
Black	4	1.8%	6 (224)	2.7%
Chinese	0	0%	0 (224)	0%
Other	1	0.4%	1 (224)	0.4%

Table 5 Baseline characteristics of EMPOWaR trial participants

From diary returns and analysis with predefined criteria, 118 (67%) of 177 women in the placebo group and 109 (65%) of 167 women in the metformin group were deemed compliant. Subsequent analysis of metformin concentrations showed that detectable concentrations were present in the blood of 80 (61%) of 131 women in the metformin group who gave a blood sample at 36 weeks' gestation. To explore dosage, we identified the proportion of drug-taking days when 2500 mg or 2000 mg of study drug was taken. In the placebo group, for 56% of all possible tablet-taking days, the top dose of 2500 mg was taken, and for 68% of these days a dose of 2000 mg or more was taken; the corresponding values in the metformin group were 38% and 62%, respectively.

Mean birthweight at delivery was 3463 g (SD 660) in the placebo group and 3462 g (548) in the metformin group (Table 6). Mean birthweight percentile was high in both groups (table 2); the proportion of liveborn babies weighing more than the 90th percentile was similar between the placebo group and the metformin group (38 [17%] of 220 and 31 [14%] of 214 babies, respectively). The primary outcome of Z score of birthweight percentile for babies liveborn at 24 weeks or more of gestation, standardised for sex, parity and gestation at delivery, was similar between the metformin and placebo groups, and the estimated effect size of metformin on the primary outcome was non-significant (Table 6).

		Placebo		Metformin				
Primary outcome	**Mean (n)**	**SD**	**Mean (n)**	**SD**	**Adjusted mean difference**	**95 % CI**	**p-value**
Z score of birthweight percentile[5]	0.2680 (220)	1.0055	0.2464 (214)	1.0179	-0.029	-0.217, 0.158	0.76
Birth outcome (all births)	**Mean or number (n)**	**SD or %**	**Mean or number (n)**	**SD or %**	**OR[6]**	**95 % CI**	**p-value**
Live birth at ≥ 24 weeks' gestation	220 (222)	99.1%	214 (221)	96.8%			
Stillbirth at ≥ 24 weeks' gestation, miscarriage or termination of pregnancy	2[7] (222)	0.9%	7[8] (221)	3.2%	3.597	0.739, 17.504	0.11
Birth outcome (liveborn babies at ≥ 24 weeks' gestation)							

[5] Centile by gestational age, sex and parity for live births at ≥ 24 weeks gestation
[6] Post hoc analysis

[7] Two terminations of pregnancy, one for fetal abnormality (split hand and foot syndrome) and one following spontaneous membrane rupture at 18 weeks gestation.

[8] Of the two stillbirths, one was at 31 weeks of a baby with a known cardiac anomaly and severe hydrops, the other was an intrauterine death of a normally formed baby born at 38 weeks with a birthweight less than the 3rd centile for gestation. Of the four miscarriages, one occurred after a road traffic accident, the other three were spontaneous. One termination of pregnancy was performed after a diagnosis of trisomy 21. None of the women returned diaries nor provided a blood sample for analysis of metformin.

Gestational age at delivery (days)	275.9 (220)	15.9	276.6 (214)	11.7
Male sex	109 (220)	49.5%	109 (214)	50.9%
Birthweight at delivery (g)	3463 (220)	660	3462 (214)	548
Birthweight percentile	57.3 (220)	27.9	56.9 (214)	28.6

Table 6 Primary and birth outcome of participants in EMPOWaR trial, intention to treat analysis.

Data are presented as mean +/- SD or %.

We recorded no evidence of a reduction in the main secondary outcome of HOMA-IR at 36 weeks' gestation, nor any evidence of a clinically or statistically significant effect of metformin on fasting or 2 h glucose (after a 75 g oral glucose challenge) or fasting insulin at 36 weeks' gestation (Table 7). By contrast, fasting glucose and HOMA-IR score at 28 weeks' gestation was lower in women in the metformin group than in those in the placebo group (supplementary tables). Metformin had no significant effect on the anthropometric variables of maternal weight gain in pregnancy or neonatal ponderal index (Table 7).

	Placebo		Metformin				
	Mean (n)	SD	Mean (n)	SD	Adjusted mean difference/ratio	95 % CI	p-value
Maternal biochemistry at 36 weeks gestation							
Fasting glucose (mmol/l)	4.42 (151)	0.48	4.35 (143)	0.45	-0.060	-0.163, 0.043	0.25
2h glucose (mmol/l) [9]	5.96 (148)	1.46	5.70 (142)	1.32	-0.251	-0.565, 0.062	0.12
Fasting insulin (µIU/ml)	30.09 (131)	13.12	32.79 (127)	24.55	1.005	0.901, 1.120	0.93
HOMA-IR score[10]	5.98 (131)	2.89	6.30 (123)	4.78	0.974	0.865, 1.097	0.67
CRP (mg/L)	9.20 (150)	7.10	7.47 (140)	4.62	0.860	0.743, 0.996	0.04
Cholesterol (mmol/L)	6.32 (144)	1.44	6.33 (139)	1.74	1.004	0.954, 1.056	0.88
HDL (mmol/L)	1.70 (145)	0.38	1.76 (138)	0.43	0.051	-0.040, 0.142	0.27
LDL (mmol/L)	3.57 (126)	1.13	3.77 (118)	1.25	1.064	0.982, 1.152	0.13
Triglycerides (mmol/L)	2.79 (146)	0.84	2.76 (140)	0.88	0.993	0.926, 1.064	0.83
IL-6 (mmol/L)	3.86 (131)	4.10	2.93 (127)	1.37	0.847	0.754, 0.952	0.01

---

[9] After a 75g oral glucose challenge
[10] Fasting glucose (in mmol/l) x insulin (µIU/ml)/22.5

Leptin (ng/ml)	105.0 (131)	52.4	106.6 (127)	58.8	1.005	0.902, 1.120	0.93
Serum cortisol (nmol/L)	821.7 (131)	232.9	867.0 (127)	225.5	1.062	0.999, 1.128	0.05
NEFA (mmol/L)	0.47 (131)	0.18	0.46 (127)	0.19	0.947	0.859, 1.044	0.27
PAI1/PAI2 ratio	3.20 (131)	2.61	2.97 (128)	2.79	0.913	0.771, 1.081	0.29
Cord blood biochemical outcomes							
Glucose (mmol/l)	3.89 (79)	1.24	4.06 (74)	1.08	1.067	0.974, 1.170	0.16
Insulin (µIU/ml)	10.95 (47)	7.49	11.41 (57)	8.80	1.060	0.767, 1.463	0.72
HOMA-IR score[10]	1.92 (38)	1.39	1.91 (41)	2.00	1.012	0.701, 1.462	0.95
CRP (mg/L) [11]	4.32 (78)	19.55	2.36 (73)	2.29			0.74
Anthropometric variables							
Maternal weight gain during pregnancy (kg)	7.23 (156)	4.91	6.70 (143)	6.00	-0.680	-1.863, 0.503	0.26
Ponderal index (mass [g] / height^3 [cm]) (live births only) [12]	2.60 (143)	0.41	2.67 (130)	0.50	1.032	0.996, 1.069	0.08

Table 7 Secondary outcomes of participants in EMPOWaR trial, intention to treat analysis.

Data are presented as mean + SD.

---

[11] Kruskal–Wallis non parametric test used

[12] Outliers outside +/- 6SD were removed, and data log-transformed for the statistical analysis, and results back transformed for this table.

Note, all parameters with the exception of maternal glucose and HDL, and neonatal CRP were log-transformed for the statistical analysis, and converted back to original scale for this table

Plasma IL-6 and CRP concentrations were both significantly lower in women given metformin, but no differences were shown in other biochemical outcomes (Table 7). Metformin did not seem to prevent gestational diabetes, as proportions of women fulfilling either IADSPG (Table 8) or WHO (data not shown) criteria for gestational diabetes at any time in pregnancy were similar between the two groups (Table 8). Furthermore, metformin did not delay the onset of gestational diabetes (IADPSG criteria): 26 women in the placebo group were diagnosed at 28 weeks' gestation and ten women were diagnosed at 36 weeks compared with 11 women diagnosed at 28 weeks and 15 women at 36 weeks in the metformin group (p=0.0718, Mantel-Haenszel χ.; post-hoc analysis).

Maternal symptoms of diarrhoea and vomiting were more common in women in the metformin group (Table 8). The incidence of other adverse outcomes, including preterm birth and low birthweight, caesarean section, and postpartum haemorrhage were similar in the two groups (Table 8). We recorded no adverse effects of metformin in post-hoc safety analyses comparing the proportion of women with a recordable serious adverse event between the two groups (Table 8). The increase in the combined adverse outcome of miscarriage, termination of pregnancy, stillbirth or neonatal death in women in the metformin group was not significant (Table 6). Admission to the neonatal unit was less common in the metformin group than the placebo group (Table 8). We noted no differences in outcomes at other time-points between the two groups (supplementary tables), with the exception of fasting glucose and HOMA-IR score, as mentioned above.

	Placebo		Metformin				
	Number (n)	%	Number (n)	%	OR	95 % CI	p-value
Women or their babies with a recorded serious adverse event	41 (222)	18.5%	37 (225)	16.4%	0.869	0.533, 1.417	0.57
Maternal delivery and postnatal							
Any caesarean section in index pregnancy	76 (222)	34.2%	65 (219)	29.7%	0.811[13]	0.543, 1.211	0.31
Primary caesarean section	46 (222)	20.7%	42 (219)	19.2%	0.908	0.569, 1.449	0.69
Postpartum haemorrhage > 1000ml	21 (216)	9.7%	20 (212)	9.4%	0.967	0.508, 1.842	0.92
Preterm birth[14]	14 (220)	6.4%	18 (214)	8.4%	1.345	0.651, 2.777	0.47
Development of gestational diabetes[15]	36 (153)	23.5%	26 (142)	18.3%	0.728	0.414, 1.283	0.27
Pregnancy induced hypertension	14 (222)	6.3%	21 (221)	9.5%	1.56	0.772, 3.152	0.22
Pre-eclampsia	3 (222)	1.4%	7 (221)	3.2%	2.39	0.61, 9.36	0.21
Fetal and neonatal outcomes (live births only)							
Admission to the neonatal unit	29 (219)	13.2%	14 (213)	6.6%	0.461[13]	0.236, 0.899	0.02

---

[13] Post hoc test

[14] Live births only; 4/14 preterm births in the placebo group and 3/18 in the metformin group were spontaneous preterm births following preterm labour.

[15] IADPSG criteria: Fasting glucose >= 5.1 mmol/l or 2hr glucose >= 8.5 mmol/l on either 28 and 36 weeks

Congenital anomaly	8 (217)	3.7%	7 (209)	3.3%	0.905[13]	0.322, 2.543	0.85
Neonatal death in the delivery room	0 (220)	0%	0 (214)	0%			
Neonatal death at a later stage	2 (220)	0.91%	1 (214)	0.47%			1.000[13 16]
Incidence of low birthweight <10th centile	11(220)	5.0%	14(214)	6.5%	1.330	0.590, 2.999	0.49
Incidence of low birthweight <3rd centile	3 (220)	1.4%	3 (214)	1.4%			1.000[16]

---

[16] Fisher's Exact test reported

	Placebo		Metformin				
	n=198		n=199				
Maternal symptoms up to 36 week gestation [17]	**Number**	**%**	**Number**	**%**	**OR**	**95 % CI**	**p-value**
Taste disturbance	32	16.2%	25	12.6%	0.745	0.424, 1.311	0.31
Skin reactions	39	19.7%	36	18.1%	0.900	0.545, 1.489	0.68
Abdominal pain	42	21.2%	49	24.6%	1.213	0.759, 1.940	0.42
Flatulence	44	22.2%	51	25.6%	1.206	0.760, 1.915	0.43
Constipation	57	28.8%	57	28.6%	0.993	0.643, 1.534	0.98
Diarrhoea	37	18.7%	83	41.7%	3.113	1.975, 4.908	<0.0001
Nausea	79	39.9%	97	48.7%	1.432	0.962, 2.132	0.078
Vomiting	43	21.7%	63	31.7%	1.670	1.064, 2.621	0.03
Headache	66	33.3%	65	32.7%	0.970	0.638, 1.474	0.89

Table 8 Adverse outcomes of participants in EMPOWaR trial, intention to treat analysis.

Data are presented as mean + SD.

---

[17] For all symptoms, categories are none/mild/moderate or severe. If a participant had any symptom mild, moderate or severe, at any time this is recorded as "yes".

Further analyses of the data on a per-protocol basis resulted in similar findings to the modified intention-to-treat analysis, with the exception of vomiting and CRP concentration, in which the direction of differences was maintained but the results were no longer significant (supplementary tables), and in 2 h glucose (estimated mean difference –0312 mmol/L, 95% CI –0.620 to –0.004; p=0.0471) and fasting insulin (6.04 pmol/L, 5.40–6.78; p=0.0173) at 28 weeks' gestation, which were significantly lower in the metformin group than in the placebo group.

3.4. Discussion

To our knowledge, EMPOWaR is the first trial of a pharmacological intervention to reduce the risk of ill health in later life, using birthweight as a surrogate marker, in the off spring of obese pregnant women. By contrast with our original hypothesis, metformin given at a median dose of 2000 mg daily to obese and severely obese pregnant women (mean BMI 37.7 kg/m.) without diabetes, from 12–16 weeks' gestation until delivery, had no effect on birthweight or neonatal or maternal anthropometry. On the basis of the study being powered to detect a clinically meaningful effect size, we conclude that this finding shows a true absence of effect of metformin on birthweight rather than a type 2 error. The absence of effect was apparent in both intention-to-treat and per-protocol analyses. We conclude that metformin does not have a role in reducing the birthweight of offspring of obese pregnant women.

The strengths of this study are its multicenter randomised controlled design, making the study robust and generalisable, and that, despite women's natural reluctance to take medication during pregnancy, we were able to recruit to our target sample size, generating adequate power to address our hypothesis.

Further detail of the subjects who were not included in the final analysis is as follows: in the placebo group there were two terminations of pregnancy, one for fetal abnormality (split hand and foot syndrome) and one following spontaneous membrane rupture at 18 weeks' gestation. In the metformin group, of the two stillbirths, one was at 31 weeks' gestation of a baby with a known cardiac anomaly and severe hydrops, the other was an intrauterine death of a normally formed baby born at 38 weeks' gestation with a birthweight less than the 3rd centile for gestation. Of the four miscarriages, one occurred after a road traffic accident, the other three were spontaneous. One termination of pregnancy was performed after a diagnosis of trisomy 21. Although there are significantly more 'non analysed' subjects in the active treatment group, none of these subjects fulfilled the eligibility criteria for

compliance and were therefore not included in the per-protocol analysis. This is reassuring from a treatment safety perspective. Additionally, there was no significant difference in the incidence of the combined adverse outcome of miscarriage, termination of pregnancy, stillbirth or neonatal death.

The eligibility criteria for adherence with treatment was determined in advance of commencing the trial and stated in the pre-publication statistical analysis plan. Adherence was calculated as follows: the number of weeks that a patient was pregnant within the study period was calculated using the gestation at baseline and the gestation at delivery. This value was then halved and compared to the number of weeks recorded in the diary. If the patient had fewer weeks of diary entries than the total eligible weeks of gestation halved then she was deemed noncompliant. If a patient had equal or more weeks diary entries than halved total weeks, she needed to have taken at least one pill on at least four days to declare a compliant week. Finally to be treatment compliant the patient needed to have equal to or more than 50% of compliant weeks out of all the available weeks. This definition was felt to reflect what was likely to happen in clinical practice[114] and thus ensure the results were generalisable to a real patient population.

Although adherence to the intervention was lower than anticipated, this was balanced by the SD for birthweight also being lower. As such, the 95% CI for the primary comparison in both the intention-to-treat and per-protocol analyses both exclude the prespecified minimum clinically relevant effect size of 0.33. We conclude that the failure to detect a significant difference between the groups is a strong negative finding rather than a result of the trial being underpowered.

We used a starting dose of metformin of 500mg, a maximum dose of 2500mg and up titrated by 500mg per week. In clinical practice, most clinicians up titrate more quickly. It is possible a different dosing regime may have produced a different result. However, a higher dose and faster up titration rate may have caused more side

effects and increased non-adherence rate or participant drop-out. The mean dose of 2000mg in the active treatment group is comparable to that used in clinical practice and this is an effective dose for the treatment of GDM so is likely to have had its expected biological effect.

Despite the lower than expected adherence, we believe that metformin still had its expected pharmacodynamic effects. Fasting glucose and insulin were lower in the metformin group than the placebo group at 28 weeks in the intention-to-treat analysis, and fasting and 2 h glucose, insulin, and HOMA-IR were all lower in the metformin group at 28 weeks in the per-protocol analysis. The subsequent lack of effect of metformin at 36 weeks is initially surprising, but might be a reflection of the changes in glucose homoeostasis throughout pregnancy in obese women. This is discussed further in Chapter 4 where insulin sensitivity is examined in detail in a subset of participants, using the hyperinsulinaemic euglycaemic clamp.

The evaluation of the efficacy of metformin in preventing GDM was not an end point in our study. Our primary interest was in its efficacy as a preventer of excessive birthweight as this is a more clinically relevant end-point in terms of future life-risk to the child of developing obesity or metabolic syndrome. Post-hoc analysis of GDM diagnosis by two commonly used criteria (IADOSG and WHO) suggested a trend towards reducing risk of GDM and later development of GDM but this did not reach statistical significance. Studies using development of GDM as a primary outcome are subject to confounding, particularly multicenter studies, due to wide discrepancies in diagnostic criteria, types of test employed, gestation of testing, and clinical management of GDM.

Although metformin had no effect on the primary outcome, the metformin-associated reduction in inflammatory markers CRP and IL-6 might be beneficial. These markers are found at higher concentrations in obese pregnant women than in pregnant women of a normal weight[104] and have been associated with adverse outcomes such as

preterm birth and pre-eclampsia[116, 117]. Our findings are consistent with those in non-pregnant individuals, in whom metformin reduces concentrations of CRP[118] and (variably) IL-6[119].

Studies of other interventions aimed at reducing birthweight in obese pregnant women, including diet and lifestyle interventions[66, 97, 115], have likewise shown no significant effects. Our data showing that metformin has no effect on birthweight in obese and severely obese pregnant women are in line with secondary outcome data from a smaller study of metformin in non-obese (mean 29.5 kg/m. [SD 7.0]) pregnant women with a history of polycystic ovary syndrome[83]. At the time of publication, we were aware of two other ongoing studies of the effect of metformin in obese pregnant women (Clinicaltrials.gov, number NCT01273584 and Australian New Zealand Clinical Trials registry, ACTRN 12612001277831). The Metformin in Obese Pregnancy study[69] has subsequently been published. Despite some differences in their trial protocol (which are discussed further in Chapter 7), the authors did not demonstrate any difference in the primary outcome of birthweight centile.

Absence of efficacy of metformin in reducing mean birthweight, despite lowering maternal glucose and insulin in mid-pregnancy, casts doubt on the 1952 Pedersen hypothesis[120] that maternal hyperglycaemia drives fetal hyperglycaemia, and hence fetal hyperinsulinaemia and fetal overgrowth. Other investigators have hypothesised, by contrast with Pedersen, that excess maternal lipids might be as, or even more important than, excess maternal glucose in fetal fat accumulation, particularly in the presence of maternal obesity[121]. The present study provides the first experimental evidence that factors other than maternal glucose are important in fetal overgrowth, challenging conventional thinking about the factors linking maternal obesity and off spring macrosomia.

Metformin might have a beneficial effect on future life risk of obesity and metabolic syndrome in offspring, even in the absence of an effect on birthweight percentile. In

an animal study[122], prenatal metformin improved glucose tolerance, and reduced accumulation of body weight, and fat mass in adulthood of the off spring, despite having only marginal effects on birthweight. Additionally, in the Metformin in Gestational diabetes (MiG) study[100], children of women randomised to the metformin group had lower visceral body fat at 2 years than did children of women randomised to insulin, despite similarities in birthweight. Further follow-up of babies born to mothers in the EMPOWaR trial is planned to explore this possibility and will identify longer-term outcomes on off spring of obese women given metformin in pregnancy. In the meantime, metformin should not be used to improve pregnancy outcomes in obese women.

3.5. Supplementary tables

	Placebo		Metformin				
	Mean (n)	SD	Mean (n)	SD	Adjusted mean difference/ratio	95 % CI	p-value
Fasting glucose (mmol/l)	4.49 (184)	0.47	4.38 (175)	0.41	-0.105	-0.193, -0.016	0.021
2h glucose (mmol/l) [18]	5.85 (184)	1.20	5.58 (174)	1.32	-0.250	-0.504, 0.005	0.055
Fasting insulin (μIU/ml) [19]	27.49 (154)	14.28	26.31 (144)	19.05	0.913	0.828,1.007	0.067
HOMA-IR score[20]	5.56 (153)	3.30	5.23 (144)	4.17	0.895	0.803,0.998	0.047

Supplementary table 1 Glucose, insulin and insulin resistance at 28 weeks' gestation

	36 Weeks				3 months Post-Partum			
	Placebo		Metformin		Placebo		Metformin	
	Mean (n)	SD	Mean (n)	SD	Mean (n)	SD	Mean (n)	SD
Height (cm)	166.0 (153)	6.0	166.3 (142)	5.6	165.3 (125)	5.9	166.1 (127)	5.8
BMI calculated	40.4 (153)	5.4	40.6 (141)	4.9	37.4 (124)	5.2	38.3 (124)	5.6

---

[18] After a 75g oral glucose challenge
[19] This parameter was log-transformed for the statistical analysis, and results back transformed for this table
[20] Fasting glucose (in mmol/l) x insulin (μIU/ml)/22.5. This parameter was log-transformed for the statistical analysis, and results back transformed for this table

	Mean (n)	SD	Mean (n)	SD	Mean (n)	SD	Mean (n)	SD
Waist (cm)	120.0 (155)	13.2	119.0 (142)	11.1	109.2 (124)	12.8	109.9 (125)	13.9
Hip (cm)	130.1 (155)	12.3	131.3 (142)	11.8	127.3 (124)	12.2	128.6 (125)	13.4
Mid arm (cm)	36.5 (154)	4.9	36.5 (142)	4.4	37.1 (123)	4.7	37.4 (125)	4.4
Mid thigh (cm)	65.3 (154)	7.4	65.2 (139)	6.8	64.3 (122)	6.7	65.8 (124)	6.8
Tricep skinfold (mm)	30.4 (155)	10.3	31.3 (143)	12.0	32.2 (123)	10.8	33.4 (125)	11.4
Bicep skinfold (mm)	26.0 (155)	10.5	26.9 (143)	11.6	27.2 (123)	12.1	29.7 (125)	15.1
Subscapular skinfold (mm)	32.7 (154)	13.5	34.5 (141)	13.9	33.2 (123)	13.1	35.9 (124)	13.2
Maternal % fat [21]	46.3 (31)	4.84	47.8 (30)	4.63	47.45 (29)	4.97	48.35 (30)	5.31
Weight gain during pregnancy (kg) [22]	7.23 (156)	4.91	6.70 (143)	6.00	-0.13 (124)	6.22	0.07 (124)	9.82
Neonatal outcomes (live births only)	**At or shortly after birth**				**At 3 months post partum**			
	Placebo		**Metformin**		**Placebo**		**Metformin**	
	Mean (n)	**SD**	**Mean (n)**	**SD**	**Mean (n)**	**SD**	**Mean (n)**	**SD**
Age at which measurements made (days)	1.04 (157)	2.44	0.97 (145)	2.44	99.59 (128)	13.12	97.72 (129)	14.01
Length (cm)[24]	51.2 (150)	4.0	50..73 (139)	3.3	62.13 (124)	4.38	61.69 (125)	6.33

---

[21] Measured only in Edinburgh participants
[22] Summary stats at week 36 are a repeat from Table 3, presented here for completeness.

Head circumference (cm)	34.7 (164)	4.2	34.8 (152)	3.6	41.30 (124)	2.87	41.02 (122)	4.42
Ponderal index (mass [g] / height ^{3} [cm]) [23] [24]	2.60 (143)	0.41	2.67 (130)	0.50	2.58 (124)	0.82	2.52 (124)	1.00
Tricep skinfold thickness (mm)	14.3 (111)	20.6	16.4 (99)	27.9	22.05 (106)	10.40	24.61 (104)	11.00
Subscapular skinfold (mm)	13.5 (113)	20.4	15.7 (98)	28.0	17.00 (104)	23.95	23.11 (104)	31.33
Baby % fat [25]	12.1 (22)	5.7	12.9 (21)	4.5	25.88 (31)	6.13	23.19 (29)	5.91
Weight at this time (g)[24] [26]	3502.65 (163)	561.32	3455.18 (146)	545.08	6085.04 (128)	1276.59	5971.97 (132)	1724.20

Supplementary table 2 Maternal anthropometry at 36 weeks' gestation and 3 months postpartum

	Placebo		Metformin	
	Mean (n)	SD	Mean (n)	SD
CRP (mg/L)	10.65 (176)	7.41	9.78 (164)	6.54
IL-6 (mmol/L)	2.73 (154)	2.16	2.38 (144)	1.19
Leptin (ng/ml)	104.4 (154)	46.4	102.3 (144)	50.5

[23] Summary stats at birth are a repeat from Table 3, presented here for completeness.
[24] Outliers outside +/- 6SD were removed.
[25] Measured only in Edinburgh participants
[26] Baby weight was recorded on two occasions – at birth by delivery team (figure used for z-score calculations) and then at time of taking research measurements by research team (this second figure is shown here and is used for calculation of ponderal index).

Serum cortisol (nmol/L)	716.5 (154)	230.8	777.2 (144)	252.8
NEFA (mmol/L)	0.42 (154)	0.14	0.43 (144)	0.16

Supplementary table 3 Metabolic and inflammatory markers at 28 weeks' gestation

	Placebo		Metformin	
	n=118		n=109	
Demographics and lifestyle	**Mean or number (n)**	**SD or %**	**Mean or number (n)**	**SD or %**
Age (years)	29.6	5.0	29.8	5.6
Currently smokes	13	11.0%	13	11.9%
Currently drinks alcohol	6	5.1%	0	0%
Illicit drug use	0	0%	0	0%
Highest educational qualification				
school ≤ 16 years	37	31.4%	26	23.9%
school ≥16 years	81	68.6%	83	76.1%
At least one previous pregnancy ≥12 weeks gestation	87	73.7%	68	62.4%

Systolic blood pressure (mmHg)	119.3	11.2	117.1	11.3
Diastolic blood pressure (mmHg)	69.0	7.7	68.5	7.9
Gestation at baseline (days)	98.9.	9.0	100.0	7.9

Medical history				
Pre-eclampsia or pregnancy induced hypertension	3	2.5%	6	5.5%
Pre- pregnancy hypertension requiring treatment	1	0.8%	1	0.9%
PCOS	14	11.9%	16	14.7%
Depression requiring treatment	33	28.0%	24	22.0%
Anxiety requiring treatment	7	5.9%	7	6.4%
Family history				
Cardiovascular disease	41	34.7%	31	28.4%
Pre-eclampsia	8	6.8%	4	3.7%
Diabetes	54	45.8%	47	43.1%
Other	58	49.2%	57	52.3%

Anthropometry				
Height (cm)	166.1	6.0	165.8	5.7
Weight (kg)	103.7	17.0	104.0	15.2
BMI calculated (kg/m^2)	37.5	5.5	37.8	4.7
Waist (cm)	108.3	12.6	108.6	11.2
Hip (cm)	126.8	11.6	127.5	12.2
Mid arm (cm)	36.6	4.7	37.1	4.4
Mid-thigh (cm)	64.2	7.3	65.3	7.0
Tricep skinfold (mm)	33.3	9.4	32.6	9.7
Bicep skinfold (mm)	27.4	10.1	27.8	10.7
Subscap skinfold (mm)	35.3	11.0	34.8	11.7
Maternal % fat[27]	46.2	5.2	48.6	5.0

Blood tests recruitment visit	

---

[27] Measured only in Edinburgh participants

Fasting glucose (mmol/L)	4.42	0.36	4.41	0.37
2 h glucose (mmol/L)[28]	5.54	1.18	5.17	1.10
Fasting insulin (µIU/ml)	22.96 (101)	10.46	21.92 (92)	8.99
HOMA-IR score[29]	4.59 (101)	2.32	4.34 (92)	1.82
CRP (mg/L)	11.4	7.9	10.0	6.3
Cholesterol (mmol/L)	4.86 (117)	1.16	4.82 (108)	1.13
HDL (mmol/L)	1.67 (117)	0.38	1.64 (108)	0.39
LDL (mmol/L)	2.98 (106)	0.75	2.90 (101)	0.90
Triglycerides (mmol/L)	1.51 (117)	0.54	1.45 (108)	0.58
IL-6 (mmol/L)	2.30 (101)	1.12	2.03 (92)	1.11
Leptin (ng/ml)	90.7 (118)	46.2	99.8 (109)	39.2
Serum cortisol (nmol/L)	384.8 (101)	135.5	438.2 (92)	186.5
NEFA (mmol/L)	0.54 (101)	0.20	0.47 (92)	0.16
PAI-1 to PAI-2 ratio	1.55 (91)	1.6	2.16 (82)	6.49

[28] After a 75g oral glucose challenge
[29] Fasting glucose (in mmol/l) x insulin (in µIU/ml)/22.5

Putative father details				
Height (cm)	178.5	7.8	177.9	13.2
Weight (kg)	92.1	21.9	94.6	27.7
Ethnic origin				
White	114	96.6%	101	92.7%
Mixed	1	0.8%	2	1.8%
Asian	0	0%	2	1.8%
Black	2	1.7%	3	2.8%
Chinese	0	0%	0	0%
Other	1	0.8%	1	0.9%

Supplementary table 4 Baseline characteristics (per-protocol analysis)

	Placebo		Metformin				
Primary outcome	**Mean (n)**	**SD**	**Mean (n)**	**SD**	**Adjusted mean difference**	**95 % CI**	**p-value**

	Mean or number (n)	SD or %	Mean or number (n)	SD or %	OR[31]	95 % CI	p-value
Z score of birthweight centile[30]	0.3130 (117)	0.9781	0.3604 (108)	1.0580	0.068	-0.188, 0.324	0.60
Birth outcome (all births)	**Mean or number (n)**	**SD or %**	**Mean or number (n)**	**SD or %**	**OR[31]**	**95 % CI**	**p-value**
Live birth at ≥ 24 weeks gestation	117 (118)	99.2%	108 (108)	100%			
Stillbirth at ≥ 24 weeks gestation, miscarriage or termination of pregnancy	1 (118)	0.8%	0	0%	<0.001	<0.001, >999.999	0.96
Birth outcome (liveborn babies at ≥ 24 weeks gestation)							
Gestational age at delivery (days)	277.6 (117)	12.7	276.6 (108)	11.5			
Male sex	58 (118)	49.2%	54 (108)	50%			
Birthweight at delivery (g)	3539.0 (117)	553.9	3503.6 (108)	562.8			
Birthweight percentile	58.527 (117)	27.7	59.894 (108)	28.3			

Supplementary table 5 Primary outcome (per-protocol analysis)

	Placebo	Metformin

---

[30] Centile by gestational age, sex and parity for live births at ≥ 24 weeks gestation
[31] Post hoc analysis

	Mean (n)	SD	Mean (n)	SD	Adjusted mean difference/ratio	95 % CI	p-value
Maternal biochemistry at 36 weeks gestation							
Fasting glucose (mmol/l)	4.43(104)	0.51	4.34(93)	0.45	-0.091	-0.221, 0.040	0.17
2h glucose (mmol/l) [32]	6.04(103)	1.53	5.79(92)	1.34	-0.248	-0.643, 0.148	0.22
Fasting insulin (µIU/ml)	31.89(88)	13.40	32.59(79)	26.07	0.939	0.819, 1.075	0.36
HOMA-IR score[33]	6.36(88)	2.96	6.22(77)	4.90	0.912	0.784, 1.060	0.23
CRP (mg/L)	8.91(104)	6.39	7.48(93)	4.58	0.901	0.760, 1.070	0.23
Cholesterol (mmol/L)	6.29(100)	1.54	6.16(91)	1.88	0.974	0.913, 1.039	0.42
HDL (mmol/L)	1.71(100)	0.37	1.76(91)	0.38	0.055	-0.046, 0.155	0.29
LDL (mmol/L)	3.67(89)	1.09	3.71(80)	1.22	1.013	0.923, 1.113	0.78
Triglycerides (mmol/L)	2.79(101)	0.90	2.84(92)	0.96	1.031	0.942, 1.127	0.51
IL-6 (mmol/L)	3.66(88)	3.73	2.77(79)	1.26	0.858	0.745, 0.988	0.03
Leptin (ng/ml)	103.80(88)	55.34	101.26(79)	47.02	1.007	0.886, 1.145	0.92
Serum cortisol (nmol/L)	806.48(88)	225.00	888.39(79)	250.73	1.092	1.010, 1.181	0.03

[32] After a 75g oral glucose challenge
[33] Fasting glucose (in mmol/l) x insulin (µIU/ml)/22.5

NEFA (mmol/L)	0.47(88)	0.19	0.48(79)	0.21	1.041	0.919, 1.179	0.52
PAI1/PAI2 ratio	3.40(91)	2.65	3.31(82)	3.09	0.895	0.721, 1.113	0.32
Cord blood biochemical outcomes							
Glucose (mmol/l)	3.94(62)	1.25	4.02(54)	1.05	1.062	0.955, 1.181	0.26
Insulin (µIU/ml)	11.14(37)	7.48	12.04(45)	9.21	1.137	0.805, 1.607	0.46
HOMA-IR score[10]	1.83(32)	1.36	1.93(30)	2.19	1.066	0.720, 1.579	0.74
CRP (mg/L) [34]	4.85(62)	21.89	2.15(53)	1.82			0.80
Anthropometric variables							
Maternal weight gain during pregnancy (kg)	7.40(106)	4.56	6.85(93)	6.11	-0.339	-1.769, 1.091	0.64
Ponderal index (mass [g] / height[3] [cm]) (live births only) [35]	2.64(90)	0.42	2.63(79)	0.46	1.004	0.961, 1.049	0.85

Supplementary table 6 Secondary outcomes (per-protocol analysis)

---

[34] Kruskal–Wallis non parametric test used

[35] Outliers outside +/- 6SD were removed, and data log-transformed for the statistical analysis, and results back transformed for this table.

Note, all parameters with the exception of maternal glucose and HDL, and neonatal CRP were log-transformed for the statistical analysis, and converted back to original scale for this table

	Placebo		Metformin				
	Number (n)	%	Number (n)	%	OR	95 % CI	p-value
Women or their babies with a recorded serious adverse event	22 (118)	18.6	14 (109)	12.8	0.643	0.311, 1.331	0.28
Maternal delivery and postnatal							
Any caesarean section in index pregnancy	43 (118)	36.4	31 (108)	28.7	0.702	0.401, 1.230	0.26
Primary caesarean section	25 (118)	21.2	22 (108)	20.4	0.952	0.500, 1.811	1.0000
Postpartum haemorrhage > 1000ml	13 (118)	11.4	9 (109)	8.5	0.721	0.295, 1.763	0.51
Preterm birth[36]	4 (117)	3.4	8 (108)	7.4	2.260	0.661, 7.732	0.24
Development of gestational diabetes[37]	22 (104)	21.2	15 (92)	16.3	0.726	0.351, 1.501	0.39
Pregnancy induced hypertension[38]	11 (118)	9.3%	11 (109)	10.1%	1.092	0.453, 2.631	0.84
Pre-eclampsia[38]	3 (118)	2.5%	3 (109)	2.8%	1.085	0.214, 5.493	0.92
Fetal and neonatal outcomes (live births only)							
Admission to the neonatal unit	13 (116)	11.2	8 (108)	7.4	0.634	0.252, 1.595	0.33
Congenital anomaly	4 (115)	3.5	4 (107)	3.7	1.078	0.263, 4.421	0.92

---

[36] Live births only; 4/14 preterm births in the placebo group and 3/18 in the metformin group were spontaneous preterm births following preterm labour.
[37] IADPSG criteria: Fasting glucose >= 5.1 mmol/l or 2hr glucose >= 8.5 mmol/l on either 28 and 36 weeks
[38] As defined by the local investigator

Neonatal death in the delivery room	0 (117)	0	0 (108)	0			
Neonatal death at a later stage	0 (117)	0	0 (108)	0			
Incidence of low birthweight <10th centile	6 (117)	5.1	6 (108)	5.6	1.088	0.340, 3.482	0.89
Incidence of low birthweight <3rd centile[39]	1 (117)	0.9	1 (108)	0.9			1.0000

---

[39] Fisher's exact test reported

	Placebo		Metformin				p-value
	n=118		n=109				
Maternal symptoms up to 36 week gestation [40]							p-value
Taste disturbance	20	16.9	17	15.6	0.905	0.447, 1.835	0.78
Skin reactions	23	19.5	21	19.3	0.986	0.510, 1.905	0.97
Abdominal pain	26	22.0	32	29.4	1.471	0.807, 2.678	0.21
Flatulence	28	23.7	38	34.9	1.720	0.964, 3.069	0.07
Constipation	38	32.2	37	33.9	1.082	0.622, 1.882	0.78
Diarrhoea	24	20.3	60	55.0	4.896	2.669, 8.617	<0.0001
Nausea	46	39.0	49	45.0	1.278	0.754, 2.168	0.36
Vomiting	24	20.3	34	31.2	1.775	0.970, 3.249	0.06
Headache	40	33.4	37	33.9	1.002	0.578, 1.737	0.99

Supplementary table 7 Adverse outcomes (per-protocol analysis)

---

[40] For all symptoms, categories are none/mild/moderate or severe. If a participant had any symptom mild, moderate or severe, at any time this is recorded as "yes".

4. Chapter 4 Assessment of whole body insulin sensitivity and lipolysis

4.1. Background

Normal pregnancy is associated with marked changes in insulin sensitivity, glucose homeostasis and lipid and protein metabolism. In early pregnancy, fasting glucose decreases by 0.11mmol/L with little further decrease by the end of pregnancy[123]. Insulin secretion increases but insulin sensitivity remains unchanged[124, 125]. This promotes lipogenesis to prepare for the rising energy needs of pregnancy and also to allow lipid storage in preparation for the energy demands of lactation. Glucose tolerance is normal at this stage, as is peripheral insulin sensitivity and hepatic basal glucose production[125-127]. By mid-pregnancy, despite the increase in insulin secretion, basal hepatic glucose production also increases, as does total gluconeogenesis to meet the increasing demands of the feto-placental unit[128-130]. By late gestation, peripheral insulin sensitivity is markedly decreased such that insulin is unable to suppress lipolysis allowing an increase in free fatty acids and therefore more energy available for gluconeogenesis[131]. Overall, the insulin sensitivity of late pregnancy is reduced by 50-70% compared to the non-pregnant state. These mechanisms are important to ensure a ready supply of energy substrates for the developing fetus.

In obese pregnant individuals, these mechanisms are disordered. Obesity is associated with a state of diminished insulin sensitivity and so obese women enter pregnancy already resistant to insulin. The reduction in fasting glucose in very early pregnancy is diminished or absent[123]. By late gestation, a physiological reduction in peripheral insulin sensitivity by 15% has been demonstrated[132]. In addition, there is marked hepatic insulin resistance with reduced insulin-mediated glucose disposal and a reduction in insulin-stimulated suppression of endogenous glucose production[133]. Thus there may be an excess of free fatty acids and glucose, which are freely transferred across the placenta and may potentially drive fetal overgrowth and

programming of later life insulin resistance. However, our own work[134] has demonstrated differences between lean and severely obese pregnant women only at early and mid-gestation, with a convergence in degree of insulin resistance by late pregnancy.

There are a variety of methods for assessing insulin sensitivity. The simplest and most basic involve measurement of fasting glucose and insulin. Indeed a single fasting glucose measurement of >7.0 mmol/L is diagnostic of diabetes[135]. A raised fasting insulin concentration is a feature of conditions associated with insulin resistance such as obesity, pre-diabetes and type 2 diabetes. Combining these two measures and using mathematical models can make a more accurate assessment of insulin resistance. Two commonly used methods are the Homeostasis Model Assessment of Insulin Resistance (HOMA-IR)[136] and the Quantitative Insulin Sensitivity Check Index (QUICKI)[137].

HOMA-IR is calculated as follows:

(Fasting glucose (mmol/L) x Fasting insulin (μU/mL)) / 22.5

QUICKI is calculated as follows:

1 / (log(fasting insulin μU/mL) + log(fasting glucose mg/dL))

Both of these methods show reasonable correlation with hyperinsulinaemic euglycaemic clamp (HEC) derived data with the major advantage of only requiring a single fasting blood sample. However, both methods assume that hepatic (fasting state) and peripheral (fed state) insulin resistance are equivalent.

The oral glucose tolerance test (OGTT) is a widely used to tool to assess glucose tolerance and insulin sensitivity. It involves the ingestion of a 75 g oral glucose load with blood samples for glucose concentration being taken immediately prior to this

and then at intervals thereafter over a 2 - 3 hour period. This can be combined with the Matsuda index[138] which includes measurements of insulin to give a more detailed measure of whole-body insulin sensitivity. It is calculated as follows:

$$10\,000/\sqrt{([\text{fasting glucose (mg/dL) x fasting insulin (}\mu U/mL)] \text{ x mean glucose}} \\ \text{(mg/dL) x mean insulin during OGTT (}\mu U/mL)]$$

For more detailed evaluation of insulin action in the post-prandial setting, minimal modeling techniques can be used, such as the frequently sampled intravenous glucose tolerance test (FSIGTT). Multiple samples are collected for measurement of glucose and insulin a mathematical model used to calculate two end point parameters: an insulin sensitivity index and glucose effectiveness.

The hyperinsulinaemic euglycaemic clamp is considered to be the gold standard technique. The basic principle of this technique is that in the fasting state, the rate of glucose production from the liver and kidneys is equal to the rate of utilisation, resulting in a relatively constant level of glucose in the blood. Insulin is then administered resulting in suppression of glucose production, stimulation of glucose uptake and consequently a fall in blood glucose. This fall in blood glucose is countered by an infusion of exogenous glucose. The rate of glucose infusion required to maintain blood glucose concentration at a specific level (usually 4.5 – 5.5 mmol/L) is an estimate of the net effect of insulin on glucose production and utilisation. Therefore, insulin sensitive subjects will require higher rates of glucose infusion compared to insulin resistant subjects. The concomitant use of stable isotope tracers allows the study of specific metabolic pathways. In the work described in this thesis, we used 6,6-d2-glucose and 1,1,2,3,3-d5-glycerol to quantify endogenous glucose production and lipolysis.

However, HEC is a reasonably invasive procedure, it is time consuming and expensive and therefore less suited to large study populations. In the EMPOWaR

study population as a whole, we used HOMA-IR as our measure of insulin resistance. In the mechanistic sub-study described in this chapter we used the hyperinsulinaemic euglycaemic clamp to assess the effect of metformin on whole body insulin sensitivity and lipolysis in a sub-group of women who were adherent to treatment. To our knowledge, this is the first study to have employed this technique to examine the effect of metformin in obese pregnant women in the context of a double-blind, randomised, placebo-controlled trial. The characteristics of women were similar to those of the EMPOWaR study overall. Importantly, those who had developed gestational diabetes were excluded.

Although metformin is widely used in pregnant women, there are no data on its mechanism of action in this scenario, nor whether is alters the physiological insulin resistance of pregnancy.

4.2. Hypothesis and aims

In this substudy we hypothesised that:

- We could use the hyperinsulinaemic euglycaemic clamp, with concomitant use of stable isotope tracers, to measure hepatic and peripheral insulin sensitivity and lipolysis at 36 weeks' gestation in obese pregnant women participating in the EMPOWaR study.
- Participants taking metformin, compared to those taking placebo, would demonstrate enhanced insulin sensitivity.

The aims of the substudy were to:

Measure hepatic and peripheral insulin sensitivity and rates of lipolysis in obese pregnant women, taking either metformin or placebo, at 36 weeks' gestation.

4.3. Methods

4.3.1. Patient recruitment, inclusion and exclusion criteria

We recruited a subset of women participating in the EMPOWaR trial. All women participating in the trial at the Edinburgh centre were invited to take part. Inclusion and exclusion criteria at baseline were the same as those for main trial (see Chapter 2). Women who had developed gestational diabetes or taken corticosteroids during their pregnancy were excluded.

4.3.2. Participant preparation

Participants (n=21) attended the Clinical Research Facility at the Royal Infirmary, Edinburgh at 0800h following an overnight fast of 8-10 hours. Height and weight measurements were recorded and fat free mass was measured using air displacement plethysmography (Bod Pod, Cosmed, www.bodpod.com). A 45mm 17 gauge cannula with a three-tap for sample collection was inserted into the superficial vein in the dorsum of one hand and kept patent with a slow infusion of 0.9% saline. This hand was wrapped in an electric heated blanket to arterialise venous blood for sample collection. A second cannula was placed in the antecubital fossa vein of the contralateral arm for administration of the infusates. Baseline blood samples were obtained for determination of baseline tracer enrichment, fasting plasma glucose, insulin and non-esterified fatty acids (NEFA).

4.3.3. Drug preparation and dosage calculations

Soluble insulin (Actrapid®, Novo Nordisk) was prepared in 0.9% saline at a concentration of 0.3U/mL. Insulin was infused at $20mU/m^2/min$ and $40mU/m^2/min$, according to body surface area (BSA) of the subject. BSA was calculated using the Mostellar formula:

$$BSA\ (m2) = \sqrt{\frac{weight\ (kg)\,x\ height\ (m)}{3600}}$$

The rate of infusions in mL/min of stock insulin solution was calculated as follows:

$$Rate\ ml/min = \frac{(20\ or\ 40)\,x\ BSA\ x\ 60}{1000\ x\ 0.3}$$

Stock solutions of the stable isotope tracers 1,1,2,3,3-$^2H_5$-glycerol and 6,6-$^2H_2$-glucose (Cambridge Isotope Laboratories, Inc., Andover, USA) were prepared by Alistair Millar and Clint Waight (Senior Radiopharmacists, Royal Infirmary of Edinburgh), using water as dilutent. D5-glycerol was provided at a concentration of 40mg/mL and d2-glycose at a concentration of 350mg/mL. Both tracers were prepared on the day of the study as a single infusion in 300mL of 0.9% saline for infusion at a rate of 50ml/hour. The molar weight of d5-glycerol is 97g/mol and d2-glucose is 182g/mol. D5-glycerol was prepared at a concentration of 0.132μmol/kg/ml to achieve a delivery rate of 6.6 μmol/kg/hour; d2-glucose was prepared at a concentration of 0.44μmol/kg/ml to achieve a delivery rate of 22μmol/kg/hour. Infusion was prepared as follows in the final volume of 300ml:

d5-glycerol: 0.132μmol/kg/ml = 12.8μg/kg/ml = 3.84mg/kg in 300ml

d2-glucose: 0.44μmol/kg/ml = 80μg/kg/ml = 24mg/kg in 300ml

4.3.4. Clamp protocol

A priming dose of d5-glycerol (1.6μmol/kg) and d2-glucose (25μmol/kg) was administered at time 0 minutes, followed by continuous infusion of the tracers as described above (6.6μmol/kg/h d5-glycerol and 22μmol/kg/h d2-glucose) for 5.5 hours. During the first 90 minutes, tracers were infused alone. From 90-210 minutes, insulin was infused at a dose of 20mU/m^2/min in order to suppress endogenous glucose production and lipolysis. From 210-330 minutes, the dose of insulin was increased to 40mU/m^2/min (to stimulate glucose uptake). Four steady state blood samples were collected at 10 minute intervals at the end of three time periods as

follows: 60, 70, 80, and 90 minutes; 180, 190, 200, 210 minutes; and 300, 310, 320, 330 minutes. Samples were placed on ice then centrifuged and stored at -80°C for analysis of glucose, glycerol and their isotopologues, insulin and NEFA. Following commencement of the insulin infusion at 90 minutes, whole blood glucose was checked every five minutes from the sampling cannula using a bedside machine (AccuCheck®, Roche, UK). Dextrose 5% was infused as required to maintain arterialised blood glucose between 4.5 and 5.5 mmol/L. Additional blood samples were obtained every 30 minutes using fluoride oxalate anticoagulant for formal enzymatic measurement of plasma glucose. A diagrammatic summary of the clamp protocol is shown in Figure 3 .
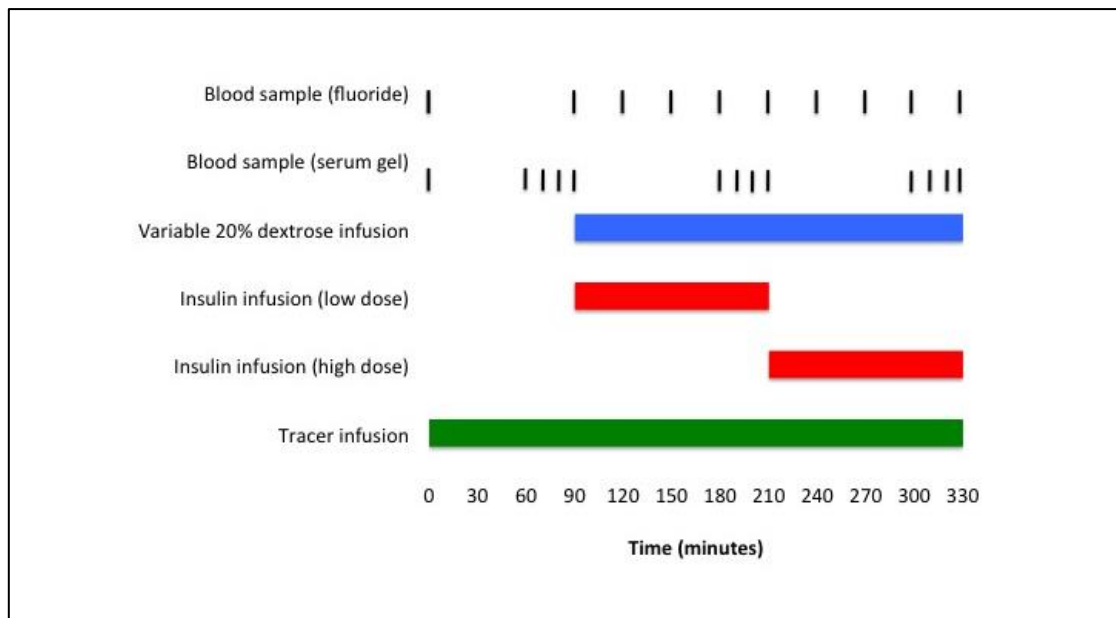

Figure 3 Summary of clamp protocol

4.3.5. Calculations

Mean glucose disposal (M) under steady state conditions was calculated in mg per kg fat free mass per minute according to the volume of glucose infused in the last 30 minutes of each steady state period. Whole body glucose disposal (WGD) is equivalent to M during the high-dose insulin phase of the clamp. An index of insulin

sensitivity, glucose disposal per unit plasma insulin (M/I), was calculated by dividing M by the mean insulin concentration (mU/L) during each steady state period. This allows correction for slight differences in achieved plasma insulin in each group.

Rate of appearance (Ra) and rate of disappearance (Rd) of glucose and glycerol were calculated using Steele's steady state equation:

$$Ra = Rd = (F/TTR\ plasma\ tracer - F)$$

where F is the infusion rate of tracer and TTR is the tracer:tracee ratio.

Endogenous glucose production (EGP) was calculated by subtracting the variable glucose infusion rate from the calculated Ra glucose. Data from glucose infusion studies were corrected for background ^{13}C enrichment. No exogenous unlabelled glycerol was infused and the abundance of other isotopic species within the tracer infusion is negligible therefore no such corrections were applied in calculation of Ra glycerol.

4.3.6. Mass spectrometry analysis

Stable isotope enrichment was measured using gas chromatography mass spectrometry (GCMS). Standard curves were prepared for concentrations of glucose, glycerol, d2-glucose and d5-glycerol in plasma with internal standards $^{13}C_6$-glucose (Isotec; Dorset, UK), and butanetriol according to standard procedure[139]. Standard enrichment curves for glucose and glycerol with d2-glucose and d5-glycerol respectively, were also prepared. Samples and standards were prepared in acetonitrile (Sigma Aldrich, Dorset, UK), internal standards added and incubated for 20 minutes, extracts collected under vacuum, eluates dried and incubated with pyridine:acetate anhydride (200 µl,1:1, v/v) before drying again, and reconstituted in 5% acetic anhydride in heptane. These were analysed on a Quantum Ultra GC-MS/MS,

operated via Xcalibur software (Version 3.0.63, ThermoFisher Scientific, Hemel Hempstead, UK) using a HP-Innowax column (30m x 0.32mm x 0.25□m; Agilent technologies Ltd., Stockport, UK). Monitored ions were the glycerol triacetate *m/z* 217, d5-glycerol triacetate *m/z* 222, butanetriol triacetate *m/z* 231 (internal standard), glucose pentacetate *m/z* 287, d2-glucose pentacetate *m/z* 289, and $^{13}C_6$-glucose pentacetate m/z 293 (internal standard).

Adherence to treatment was determined by measurement of metformin in plasma using an Aria-TSQ Quantum LC-MS/MS liquid chromatography tandem mass spectrometer (ThermoFisher Scientific, Hemel Hempstead, UK). Metformin was extracted from plasma (100 μL) using an SLE+ plate (Biotage, Ystrad Mynach, UK) following enrichment with d6-metformin (200 ng) as internal standard (IS). Calibration standards ranged from 0.5-1000 ng metformin. Analytes were eluted, reduced to dryness under nitrogen (40^0C) and reconstituted in water/acetonitrile (100 μL; 80:20 v/v)). Analysis was carried out by liquid chromatography tandem mass spectrometry (LC-MS/MS). Chromatographic separation was achieved using an Aria CTC autosampler and Allegros pump on an ACE Excel Super2C18 column (100x3 mm; 2 μm, HiChrom, UK) protected by a Kinetex KrudKatcher® (Phenomenex, UK) and detected on a TSQ Quantum Discovery triple quadrupole MS (Thermo Fisher Scientific, Hemel Hempstead, UK) operated by selective reaction monitoring in positive electrospray ionization mode (300^oC, 3 kV). The mobile phase was 0.1 % formic acid (FA) in water (A), 0.1 % FA in acetonitrile (B) at a flow rate of 0.2mL/min, 30^0C. Gradient elution was achieved by increasing the percentage of acetonitrile from 20 to 90% over a 5 minute run time. Metformin and its isotopically labelled internal standard eluted at 2.1 minutes. Transitions monitored for were *m/z* 130.1 → 60.1, 71.1 and *m/z* 136.2 → 60.1, 71.1 for metformin and IS, respectively. Linear regression analysis of calibration standards, calculated using peak area ratios of metformin to IS, was used to determine the concentration of metformin in the samples.

4.3.7. Laboratory analysis

Glucose concentrations were measured by a hexokinase method (Abbot Architect platform, Illinois, USA). NEFA were measured by colorimetric methods (Wako Chemicals, Neuss, Germany). Serum samples for insulin were analysed by ELISA (Demeditec Diagnostics, Kiel, Germany).

The candidate carried out the following tasks: participant recruitment, glucose tolerance testing, air displacement plethysmography measurements and all clamp studies. Sanjay Kothiya and Natalie Homer, Wellcome Trust Mass Spectrometry Core Facility, measured stable isotope enrichment and metformin enrichment respectively. Dr Ruth Andrew reviewed all of the mass spectrometry data and calculations. Serum insulin and NEFA were analysed by Linda Nicol (senior laboratory technician, QMRI). The clinical biochemistry laboratory at the Royal Infirmary of Edinburgh performed the plasma glucose measurements.

4.3.8. Statistical analysis

Data are expressed as mean $\pm$ SEM unless otherwise stated. Comparisons were made between groups using unpaired Student's t-test. HOMA-IR data were log transformed to achieve normal distribution and results back transformed for reporting. Glycerol steady state data could not be transformed into a normal distribution and were therefore compared using Mann Whitney test. Significance was set at $p<0.05$. No adjustment was made for multiple comparisons. GraphPad Prism software (version 6.0) was used for statistical analysis. All statistical analyses were carried out by the candidate.

4.4. Results

Of the 119 participants randomised to the main trial in the Edinburgh site, 21 remained eligible and willing to participate in this sub-study at 36 weeks' gestation. In one participant, intravenous access failed during the procedure and could not be re-established and so data were not acquired. Hence data were obtained from 20 of the 21 subjects who attended for a clamp study. Clamp studies were performed blind to treatment allocation. End-of-study unblinding revealed final cohort numbers as follows; placebo group, n=11; metformin group, n=9. All participants (placebo and metformin groups) were compliant with their study medication by diary entries. One woman in the metformin group did not have detectable levels of metformin in her blood at 36 weeks' gestation, and her data were therefore excluded from analysis. Hence the final sample size was 11 women in the placebo group and 8 women in the metformin group. This is summarised in Figure 4 .
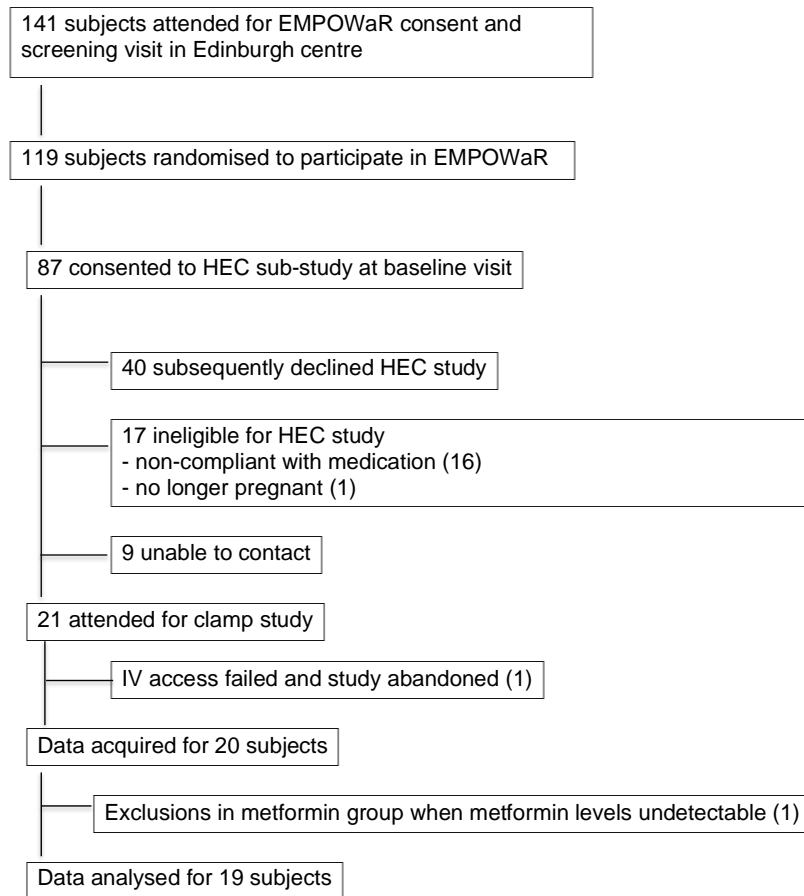
```
┌─────────────────────────────────────────────┐
│ 141 subjects attended for EMPOWaR consent and│
│ screening visit in Edinburgh centre          │
└─────────────────────────────────────────────┘
                    │
┌─────────────────────────────────────────────┐
│ 119 subjects randomised to participate in EMPOWaR│
└─────────────────────────────────────────────┘
                    │
┌─────────────────────────────────────────────┐
│ 87 consented to HEC sub-study at baseline visit│
└─────────────────────────────────────────────┘
         │
         ├──────┤ 40 subsequently declined HEC study │
         │
         │      ┌─────────────────────────────────────────┐
         ├──────┤ 17 ineligible for HEC study             │
         │      │ - non-compliant with medication (16)    │
         │      │ - no longer pregnant (1)                │
         │      └─────────────────────────────────────────┘
         │
         ├──────┤ 9 unable to contact │
         │
┌──────────────────────────┐
│ 21 attended for clamp study│
└──────────────────────────┘
         │
         ├──────┤ IV access failed and study abandoned (1) │
         │
┌──────────────────────────┐
│ Data acquired for 20 subjects│
└──────────────────────────┘
         │
         ├──────┤ Exclusions in metformin group when metformin levels undetectable (1) │
         │
┌──────────────────────────┐
│ Data analysed for 19 subjects│
└──────────────────────────┘
```

Figure 4 Consort diagram of recruitment of HEC substudy participants

Participant baseline characteristics are shown in Table 9. There were no obvious differences between those in the placebo and metformin groups and the characteristics of those in the substudy were similar to those in the EMPOWaR study as a whole.

	Placebo		Metformin	
	Mean or n	SD or %	Mean or n	SD or %
	n=11		n=8	
Age (years)	29.6	3.6	32.6	3.7
Nulliparity	n=5	46%	n=3	38%
BMI at baseline (kg/m2)	35.7	3.5	38.5	4.4
Body fat % at time of clamp (%)	46.2	5.3	49.1	3.9
Gestation at time of clamp (days)	256.9	2.1	257.3	3.4

Table 9 Demographic characteristics of participants in HEC sub-study.

Data are presented as mean + SD or %.

4.4.1. Isotopic enrichments

Isotopic enrichment of d2-glucose and d5-glycerol was achieved for both placebo and metformin groups during each steady state period, indicating technical success of the clamps (Figure 5).
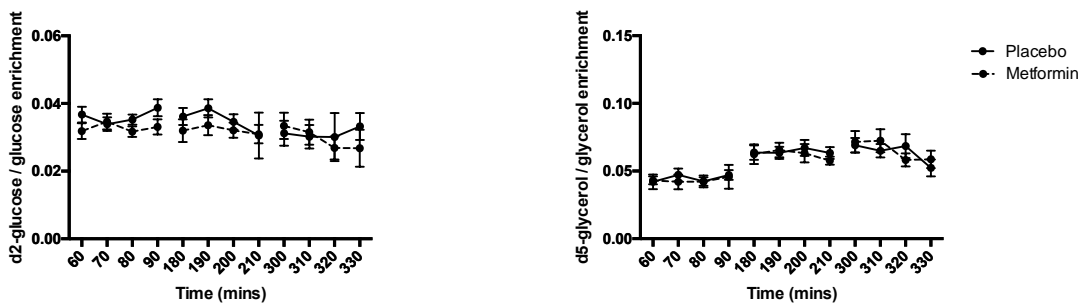

Figure 5 D2-glucose and d5-glycerol enrichments during steady state.

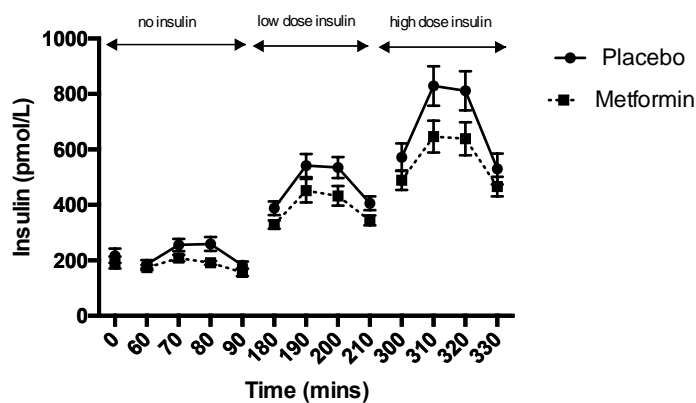**Data are presented as mean +/- SEM.**

4.4.2. Clamped glucose, insulin, glycerol and NEFA

Mean plasma glucose, insulin, glycerol and NEFA concentrations during each steady state period are shown in Figure 6 . Clamped glucose was similar in both the metformin and placebo groups (Table 10). Achieved plasma insulin concentrations were lower in the metformin group during both the low dose insulin and high dose insulin phases of the clamp (Table 10). The expected insulin-mediated suppressions of NEFA and glycerol are observed.
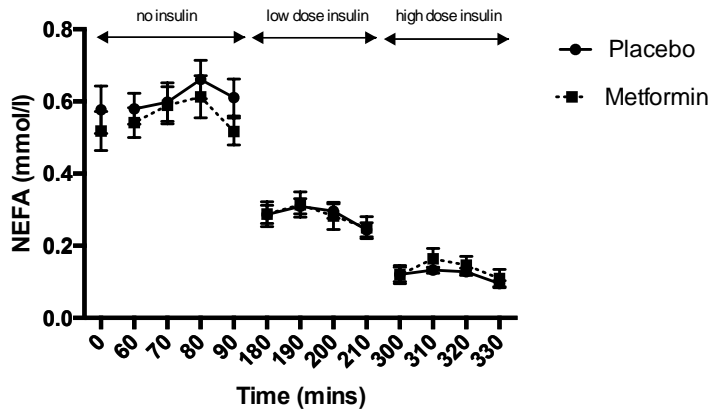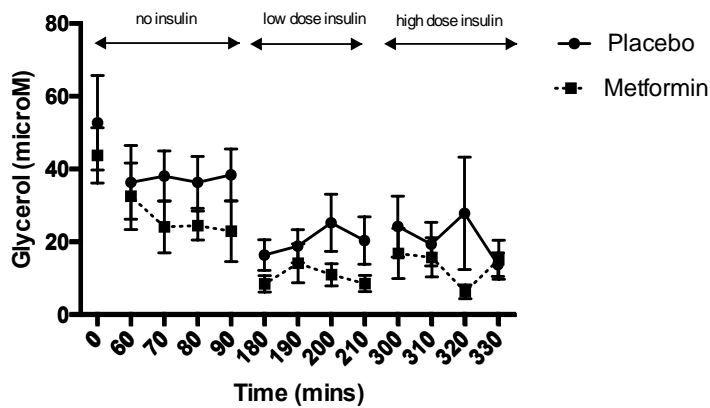
A
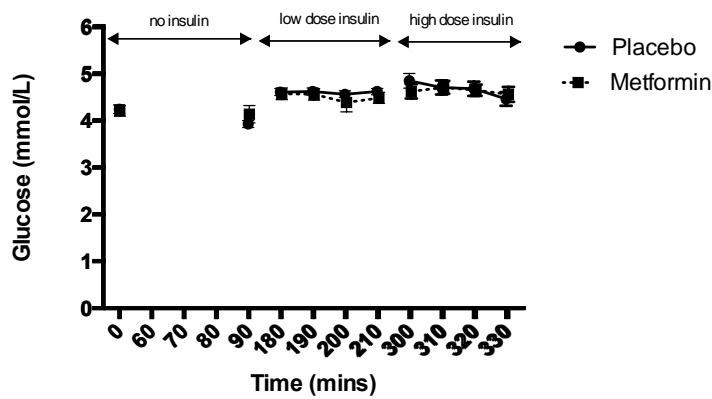
B

C

D

Figure 6 Clamped plasma glucose (A), insulin (B), NEFA (C) and glycerol (D) in placebo and metformin groups.

Data are presented as mean +/- SEM.

4.4.3. Endogenous glucose production and glucose disposal

Whole body glucose disposal (WGD) was calculated in milligrams per kilogram fat free mass (mg/kgFFM) per minute at steady state during the high dose insulin phase of the clamp. WGD is an indirect measure of insulin sensitivity, where a greater glucose disposal rate implies greater insulin sensitivity. Glucose disposal per unit plasma insulin (M/I) was also calculated to correct for slight differences in achieved plasma insulin. M/I, but not WGD, was higher in the metformin treated group compared with the placebo group (difference between means 0.02 [95% CI 0.001 to

0.03], p=0.04, and difference between means 0.78 [95% CI -0.12 to 1.67], p=0.08 respectively) (Figure 7 ,Table 10). There was no significant difference in HOMA-IR scores between the two groups (difference between means -0.87 [95% CI -3.31 to 1.57], p=0.46) (
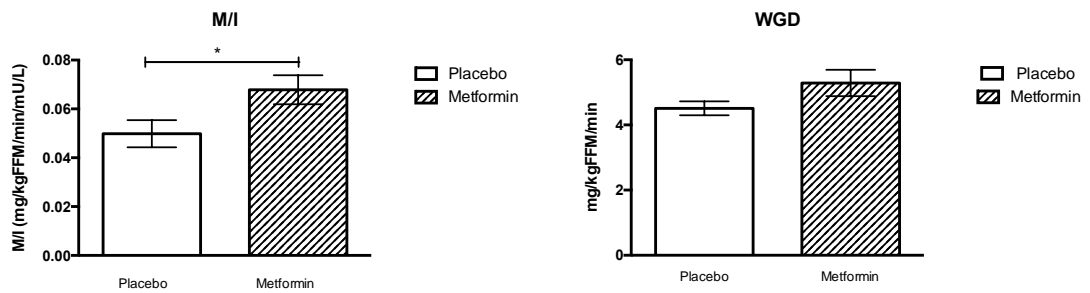
Table **10**).

Figure 7 M/I ratios and whole body glucose disposal in placebo and metformin groups. Data are expressed as mean +/- SEM. *_p_<0.05.

In the absence of insulin, the rate of disappearance of glucose (Rd) and endogenous glucose production (EGP) are equivalent. This steady state period represents fasting conditions. EGP was greater in the metformin treated group than in the placebo group in this phase of the clamp (difference between means 0.54 [95% CI 0.08 to 1.00], p=0.02). During low dose insulin infusion, EGP was again higher in the metformin treated group (difference between means 0.43 [95% CI 0.02 to 0.84], p=0.04)(Figure 8 ,Table 10). There was no significant difference in the percentage suppression from basal EGP to EGP during low dose HEC between the two groups (difference between means 2.89 [95% CI -7.65 to 13.42], p=0.57). The rate of disappearance (Rd) of glucose was also significantly greater in the metformin group compared to the placebo group during the low dose insulin phase of the clamp (difference between means 0.36 [95% CI 0.22 to 1.18], p=0.007). At high dose insulin infusion, Rd was increased further but there was no significant difference between the treatment groups (difference between means 0.35 [95% CI -0.79 to 1.50], p=0.52)(Figure 8 ,Table 10).
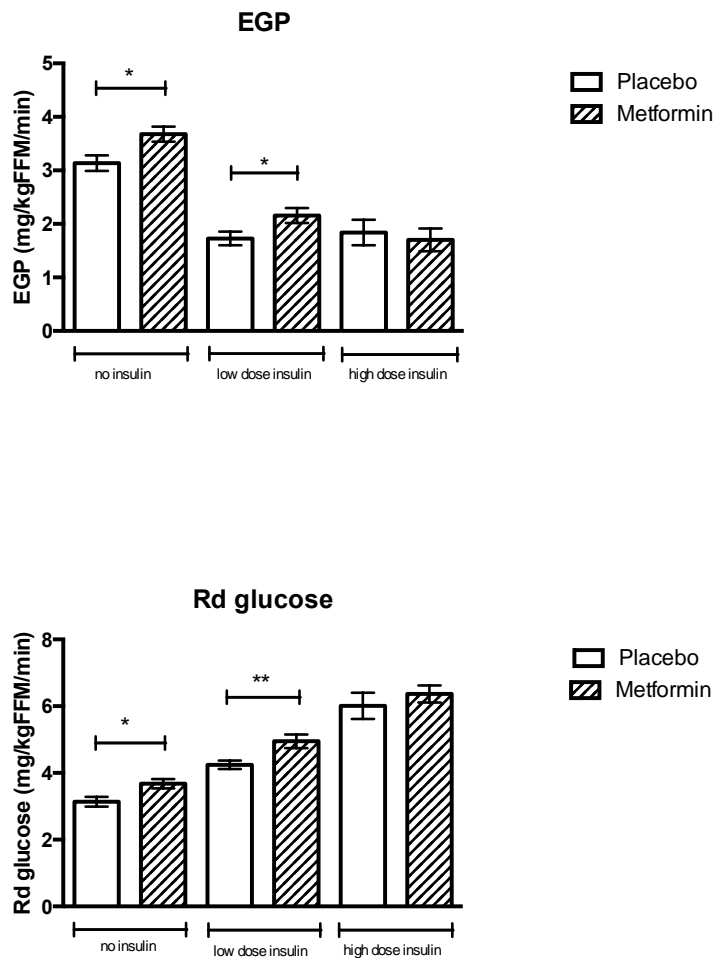
EGP

Rd glucose

Figure 8 Endogenous glucose production (EGP) and rate of disappearance (Rd) of glucose per kg fat-free mass following low dose and high dose insulin infusion in placebo and metformin groups.

Data are presented as mean +/- SEM. *$p<0.05$ **$p<0.01$.

4.4.4. Lipolysis

Glycerol turnover (or Rd glycerol) per kilogram fat free mass is shown in Figure 9 . There was no difference in glycerol turnover between the metformin and placebo treated participants (no insulin difference between means 0.03 [95% CI -0.12 to 0.18], p=0.67; low dose insulin difference between means 0.02 [95% CI -0.06 to 0.10], p=0.64; high dose insulin difference between means -0.01 [95% CI -0.10 to 0.08], p=0.87)(Figure 9 ,Table 10). Low dose insulin resulted in suppression of Rd

glycerol in both groups. High dose insulin resulted in no further suppression of Rd in either group. Participants taking metformin had lower circulating glycerol levels during the low dose insulin phase of the clamp but NEFA levels were similar between the two groups throughout the study (Table 10).
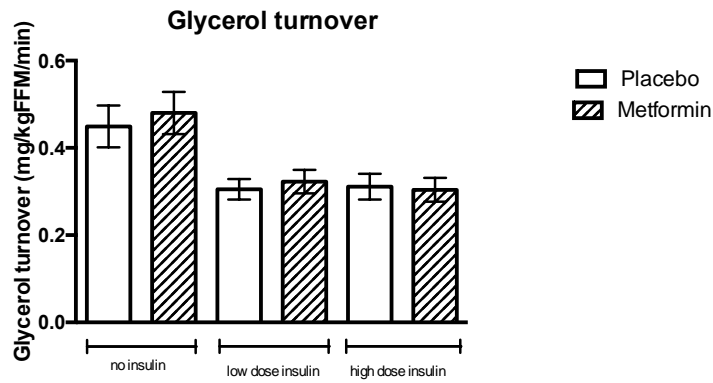

Figure 9 Glycerol turnover per kg fat-free mass following low and high dose insulin infusion. Data are presented as mean +/- SEM.

	Placebo (n=11)		Metformin (n=8)					
	Mean	SEM or IQR	Mean	SEM or IQR	Mean/median difference	95% CI	p value	Significance
Fasting pre-insulin								
Glucose (mmol/L)	4.26	0.09	4.20	0.11	-0.05	-0.35 to 0.25	0.71	
Insulin (pmol/L)	217.40	25.76	192.10	21.83	-25.30	-100.4 to 49.77	0.49	
HOMA-IR	6.04	0.89	5.17	0.58	-0.87	-3.31 to 1.57	0.46	
NEFA (mmol/L)	0.58	0.07	0.52	0.05	-0.06	-0.25 to 0.13	0.52	
Glycerol (microm/L)	52.61	12.99	43.81	7.60	-8.81	-43.92 to 26.30	0.60	
During tracer infusion without insulin								
Glucose (mmol/L)	3.94	0.07	4.13	0.18	0.19	-0.17 to 0.56	0.28	
Insulin (pmol/L)	221.20	10.88	182.40	6.13	-38.80	-66.31 to -11.29	0.006	**
NEFA (mmol/L)	0.61	0.02	0.56	0.02	-0.05	-0.12 to 0.02	0.18	
Glycerol (microm/L)	34.43[a]	19.52-60.23[b]	21.37[a]	12.07-33.25[b]	-13.06[c]	492[d]	0.05	*

EGP (mg/kgFFM/min)	3.14	0.15	3.68	0.14	0.54	0.08 to 1.00	0.02	*
Rd glucose (mg/kgFFM/min)	3.14	0.15	3.68	0.14	0.54	0.08 to 1.00	0.02	*
Ra glycerol (mg/kgFFM/min)	0.45	0.05	0.48	0.05	0.03	-0.12 to 0.18	0.67	
During low-dose insulin infusion								
Glucose (mmol/L)	4.61	0.03	4.49	0.07	-0.12	-0.27 to 0.02	0.10	
Insulin (pmol/L)	468.00	19.27	389.10	17.11	-78.85	-132.5 to -25.22	0.005	**
NEFA (mmol/L)	0.28	0.01	0.28	0.02	-0.001	-0.04 to 0.04	0.95	
Glycerol (microm/L)	15.78 [a]	6.25-28.18 [b]	9.03 [a]	2.62-14.46 [b]	-6.75 [c]	497 [d]	0.03	*
EGP (mg/kgFFM/min)	1.73	0.13	2.16	0.14	0.43	0.02 to 0.84	0.04	*
Rd glucose (mg/kgFFM/min)	4.25	0.13	4.95	0.21	0.70	0.22 to 1.18	0.007	**
M value (mg/kgFFM/min)	2.73	0.08	3.05	0.16	0.32	-0.03 to 0.68	0.07	
Ra glycerol (mg/kgFFM/min)	0.31	0.02	0.32	0.03	0.02	-0.06 to 0.10	0.64	
During high-dose insulin infusion								
Glucose (mmol/L)	4.68	0.08	4.63	0.07	-0.06	-0.27 to 0.16	0.61	

Insulin (pmol/L)	685.80	36.49	559.90	27.35	-126.0	-223.1 to -28.77	0.01	*
NEFA (mmol/L)	0.12	0.01	0.13	0.01	0.02	-0.01 to 0.04	0.25	
Glycerol (microm/L)	14.49 [a]	0.23-26.33 [b]	8.71 [a]	0.49-24.93 [b]	-5.78 [c]	631 [d]	0.44	
EGP (mg/kgFFM/min)	1.84	0.24	1.70	0.21	-0.14	-0.87 to 0.59	0.70	
Rd glucose (mg/kgFFM/min)	6.01	0.40	6.37	0.26	0.36	-0.79 to 1.50	0.52	
WGD or M value (mg/kgFFM/min)	4.51	0.21	5.29	0.41	0.78	-0.12 to 1.67	0.08	
M/I [e]	0.05	0.01	0.07	0.01	0.02	0.001 to 0.03	0.04	*
Ra glycerol (mg/kgFFM/min)	0.31	0.03	0.30	0.03	-0.007	-0.10 to 0.08	0.87	

[a]**Median,** [b]**Interquartile range,** [c]**Median difference,** [d]**Mann Whitney U,** [e]**M/I is an index of insulin sensitivity calculated by dividing M value (mg/kgFFM/min) by mean insulin concentration (mU/L) during that part of the clamp**

Table 10 Average concentrations of all analytes during steady state.
Data are presented as mean +/- SEM or median +/- IQR. *$p<0.05$, **$p<0.01$.

4.5. Discussion

We hypothesised that administration of metformin to obese pregnant women would increase hepatic and peripheral insulin sensitivity when measured in the third trimester. In support of this, we have shown that subjects taking metformin demonstrated greater insulin stimulated glucose disposal (M/I) and lower circulating levels of glycerol during high dose insulin infusion. These changes were not detected using HOMA-IR when measured in both the clamp participants and the larger EMPOWaR study population as a whole, where we found no difference in HOMA-IR, glucose or insulin at 36 weeks' gestation, either in the intention to treat analysis, or the per protocol analysis[140]. These data confirm that the HEC is a more sensitive measure of insulin resistance than HOMA-IR, at the end of pregnancy.

Analysis for this mechanistic substudy included all participants in the placebo group but was restricted to subjects in the treatment group with detectable levels of metformin in their blood, as measured by mass spectrometry. Any detectable level of the drug was taken as being indicative of compliance rather than a specific range of metformin concentration, as this could have been variable depending on the individuals dosing regime and rate of metabolism. This meant that one subject in the active treatment groups was excluded from analysis, as there was no detectable level of metformin in her blood sample. According to her treatment diary entries, she was eligible for inclusion in the per protocol analysis. This perhaps highlights one of the limitations of using patient treatment diary entries as our measure of compliance. However, as there is no equivalent biological measure of placebo compliance, using diary entries is the most appropriate measure of compliance.

In addition to greater insulin stimulated glucose disposal in the participants taking metformin, we observed enhanced rate of disappearance of glucose in the active treatment group, suggesting improved peripheral insulin sensitivity. However, endogenous glucose production was higher in the metformin treated subjects, suggesting that, if anything, those on metformin exhibit reduced ability to suppress

hepatic glucose production in response to insulin and enhanced glucose release on fasting. This is perhaps surprising given that metformin is though to exert its action principally via the liver, inhibiting gluconeogenesis and reducing hepatic glucose production[141], though an effect mediated via peripheral glucose disposal has also been shown[142, 143]. More recently, metformin has been shown to stimulate endogenous glucose production in healthy fasting individuals who are not pregnant[144] but its mechanism of action in pregnancy has, to our knowledge, never been fully assessed. We are aware of one other study that used the HEC to assess the effect of metformin in pregnant women with polycystic ovarian syndrome (PCOS)[145], but there was no control 'untreated' group so the independent effects of metformin could not be determined.

Our data suggest enhanced glucose flux in the metformin treated women, i.e. higher liver production and higher peripheral disposal of glucose. In normal pregnancy, insulin resistance increases by almost 50%, with most of this increase occurring in the third trimester[126]. This promotes maternal lipogenesis in preparation for breastfeeding and supports the rapid phase of fetal growth that occurs at this stage of pregnancy. A decline in peripheral insulin sensitivity (primarily as a consequence of placental lactogen) results in progressive shift from lipogenesis to lipolysis, with increased levels of free fatty acids and increased gluconeogenesis and hepatic glucose production[146], with a corresponding increase in maternal insulin secretion. In obese women, there is pre-existing insulin resistance so the existing reduced peripheral insulin sensitivity is merely exacerbated by the pregnancy. Our clamp data suggest that metformin has had some effect on improving peripheral insulin sensitivity but the increased hepatic glucose production of late pregnancy is too overwhelming for there to be any net benefit.

Interpretation of this is perhaps complicated by the presence of the feto-placental unit, which represents a significant proportion of the fat free mass at 36 weeks' gestation, is a major consumer of maternal glucose at term[147, 148], and may affect the validity of the HEC. Glucose uptake by the fetoplacental unit may act as a glucose

'sink' which could in turn lead to an overestimation of insulin sensitivity with an increased contribution of the fetoplacental unit to apparent maternal glucose disposal. It is not possible to measure the specific rate of glucose uptake by the fetus and placenta *in vivo* but data from a rat model suggest that placental glucose transport is not directly sensitive to maternal insulin concentrations[149]. With these limitations in mind, the HEC technique remains the gold standard method of assessment of insulin sensitivity in pregnant women. We assume the placenta itself does not produce glucose, although this is controversial with some studies suggesting it may produce a small amount[150]. Metformin does not appear to have a direct effect on placental glucose transport in an isolated placental cotyledon model[151] but it does cross the placenta[152, 153] and likely drives increased fetal insulin sensitivity and glucose uptake. Therefore, any potentially beneficial effects of metformin in limiting fetal weight gain by increased peripheral insulin sensitivity and glucose disposal in the mother, may be offset by the increased glucose flux, particularly across the placenta, which would have a growth promoting effect on the fetus. This balance may explain the lack of effect of metformin to prevent high birth weight in the main EMPOWaR trial[140] and in another recently published trial also testing the effect of metformin in obese pregnant women[69].

In animal studies and other patient groups metformin treatment has been associated with a beneficial effect on circulating lipid profile[154-157] although the mechanism of this effect remains incompletely understood. We found minimal effects of metformin on the lipolytic pathway, only that glycerol levels were lower in the metformin group during each phase of the clamp. Maximal suppression of lipolysis (around 70%) was achieved with low dose insulin in both treatment groups demonstrating equal sensitivity of the lipolytic pathway to insulin. There were no differences in glycerol disposal or NEFA levels between the metformin and placebo groups.

Accurate assessment of glycerol by mass spectrometry is technically challenging and this is reflected by the wide variance in measurements obtained. The rate of appearance of glycerol (which should be equal to the rate of disappearance or

glycerol turnover under steady state conditions) should be a reasonably accurate measure of lipolysis as this is the only source of glycerol. However, the technical difficulties of the assay may have limited the validity of this assessment. An alternative means of assessing lipolysis is by measurement of rate of appearance of fatty acids but this requires the use of fatty acid tracers, which are complexed with albumin and not approved for use in pregnant women.

Enhanced rates of lipolysis are thought to be increasingly important towards the end of normal pregnancy to provide maternal substrates for gluconeogenesis and triglyceride synthesis and spare glucose to facilitate normal fetal growth. Gestational hyperlipidaemia is exaggerated in obese women, compared to lean women[158] and reaches a peak in the second trimester, earlier than in lean women. Obese women appear to demonstrate less metabolic flexibility in their response to pregnancy and, as with the glucose response, perhaps these changes are so profound by the third trimester we are not seeing any effect of metformin.

Reduced third trimester lipolysis is associated with fetal growth restriction[159] so these data are perhaps reassuring on the safety of metformin in pregnancy in terms of not increasing the risk of intrauterine growth restriction. Alternatively, the lack of effect of metformin on the lipolytic pathway may be an alternative explanation for the failure of its use as an agent to limit birthweight in obese women[69, 140].

We performed these clamp studies at the end of pregnancy, when we anticipated that insulin resistance would be at its most profound[62], and any impact of an insulin sensitising agent would be greatest. In retrospect, this may be incorrect. In the full cohort of participants in the EMPOWaR study, both glucose and HOMA-IR were lower in the metformin group (compared to the placebo group) at 28 weeks' gestation, hence clamp studies at this gestation may have been more informative, with more profound differences between the groups. In support of this, previous work by our group comparing lean and obese pregnant women showed higher insulin stimulated endogenous glucose production and lipolysis and lower glucose disposal

in obese compared with lean women at 19 weeks' gestation, but not at 36 weeks' gestation[134]. It may be that by the end of pregnancy, the physiological effects of pregnancy are dominant over differences that may be induced either pathologically by obesity or pharmacologically by metformin.

In conclusion, these data confirm that metformin improves peripheral insulin sensitivity in obese pregnant women at 36 weeks' gestation. However, this may be offset by increased insulin clearance, and increased hepatic glucose production and therefore glucose flux across the placenta, potentially explaining a lack of effect on limiting excess fetal growth.

5. Chapter 5 MRI assessment of maternal and fetal body composition

5.1. Background

5.1.1. Maternal body composition

Body mass index is the most commonly used measure of nutritional status in adults. BMI describes weight relative to height and is expressed in kg/m^2. It is calculated using the following formula:

$$BMI = \text{weight (kg)} / [\text{height (m)}]^2$$

The World Health Organising categorises BMI into six bands to classify nutritional status (Table 11).

Nutritional status	BMI (kg/m^2)
Underweight	<18.5
Normal weight	18.5 – 24.9
Pre-obesity	25.0 - 29.9
Obesity class I	30.0 – 34.9
Obesity class II	35.0 – 40.0
Obesity class III	>40.0

Table 11 WHO classification of BMI

BMI is easily measured and simple to calculate which makes it a commonly used tool for large population data gathering and useful proxy for adiposity. However, a

major limitation is that it does not distinguish between fat and fat-free mass, and indeed other components of body weight. So, for example, it will overestimate adiposity in those with increased lean body mass such as athletes. It also does not account for distribution of body fat, and it is has long been evident that it is the site, rather than the total quantity of body fat that is important in determining associated risk of morbidity and insulin sensitivity[160-163].

More recently, it has also been recognised that deposition of lipid in 'ectopic' sites, namely the liver and skeletal muscle are major contributors to the development of insulin resistance[164, 165]. It remains unclear whether increasing adiposity causes the deterioration in insulin sensitivity or vice versa. The 'portal hypothesis' of obesity suggests an increase in central abdominal fat leads to elevated delivery of free fatty acids and inflammatory cytokines to the liver and consequently hepatic insulin resistance develops and drives glucose upwards[166]. The 'spillover hypothesis' suggests that in the context of obesity, the subcutaneous compartment becomes saturated and leads to the accumulation of visceral fat and deposition of lipid in ectopic sites such as the liver and muscle[167]. Clearly the two hypotheses are not mutually exclusive and it is likely both are contributory mechanisms. Regardless of the exact cause, there is no doubt that excess lipid accumulation is associated with impaired insulin sensitivity and morbidity such as type two diabetes.

Fat distribution in normal and obese pregnancy and in pregnancy associated with diabetes is less well studied and the contribution it pays to maternal and fetal outcomes and longer-term health is not clear. However, gestational weight gain is one of the most important predictors of postpartum weight retention[168] and thus contributes significantly to the obesity epidemic among young women[169]. Gestational weight gain, and its relative composition, is highly variable but in lean women the contribution from fat tends to be predominantly in the subcutaneous compartments, largely trunk and thigh[170, 171]. Obese women actually tend to gain less weight than lean women during pregnancy but the fat mass that they do gain tends to be more central and therefore potentially more metabolically harmful[172].

5.1.2. Fetal body composition

Gestational weight gain and pre-existing obesity also impact on fetal growth. Glucose is the major substrate that determines fat accumulation in the fetus, the greater the glucose supply the greater the deposition of fat[173]. There is a linear association between maternal glucose tolerance and neonatal adiposity at birth[61] and a strong positive correlation between degree of maternal insulin resistance and neonatal fat mass at birth[63]. Increasing maternal BMI has also been show to be associated with increased intrahepatocellular lipid in the newborn, assessed by MR proton spectroscopy[174].

The conventional strategy for measurement of fetal growth is typically by ultrasound. Estimation of fetal weight is based on measurement of the fetal head circumference, femur length and abdominal circumference, with the abdominal circumference being the most individually sensitive predictor of fetal macrosomia[175, 176]. The abdominal circumference is predominantly affected by the size of the fetal liver and is positively correlated with hepatic glycogen stores, which increase towards term[177, 178].

Trans-abdominal ultrasound is undoubtedly more difficult to perform and less accurate in obese subjects[179]. The adipose tissue layer, by absorbing the associated energy, attenuates the ultrasound signal. To improve depth of penetration of the ultrasound signal, a lower frequency probe is required but this sacrifices image quality.

5.1.3. Magnetic resonance imaging and spectroscopy

Magnetic resonance imaging is a technique that employs the properties of atomic nuclei within a strong magnetic field to create images. It is an excellent imaging modality for producing high-resolution images of soft tissues enabling distinction to be made easily between different tissue types. Image quality is not affected by the body habitus of the individual being scanned. Additionally, it does not involve the

use of ionising radiation, thus making it a suitable imaging modality for scanning pregnant women.

Magnetic resonance spectroscopy is an additional analytical tool, which can be used to study the metabolic properties of tissues. It produces a spectrum, as opposed to an image. The most widely used element in clinical spectroscopy is hydrogen. The behaviour of the hydrogen protons (^{1}H) in the magnetic field depends on the chemical structure of the molecule it is part of and thus characteristic spectra are generated for different metabolites. The technique allows both the identification and quantification of specific metabolites and thus we were able to quantify ectopic lipid (triglyceride) deposits in sites such as the liver and skeletal muscle, which would not be visible on standard imaging.

MRI has been used for many years in pregnant women with no apparent adverse effects on the mother or developing fetus[180, 181]. Most MRI machines in clinical use field strength of 1.5 Tesla (T). We used a 3T magnet, which affords better image quality and potentially slightly shorter scan times. However, there are fewer safety data on 3T scans in pregnancy. There are two theoretical safety concerns; exposure of the fetus to overheating and exposure of the fetus to excess acoustic noise. With regard to heat exposure, studies in a sheep model and mathematical modelling of heat exchange in a pregnant human model are reassuring[182, 183]. However, the degree of heating required to which would pose a teratogenic risk to the fetus is not known and scanning at 3T is not recommended in the first trimester of pregnancy. Our scans were performed in the third trimester where any effect is not likely to be significant. Measures taken to minimise the risk of harm were maintenance of a cool temperature in the scan room with air conditioning and a fan, and ensuring the subject wore minimal, loose fitting clothing during the scan. We also limited the scan time to 60 minutes, even if this sacrificed the acquisition of all desirable data. The second safety consideration is acoustic damage. MRI scanners are noisy and subjects are given ear protectors to wear throughout the scan. The amniotic fluid, the muscular wall of the uterus and the abdominal wall protect the fetus so the impact of noise on the fetus is

likely to be minimal. There are few long-term follow up studies on children exposed to MRI in utero but those that exist are reassuring in demonstrating an absence of harm[184-188].

5.2. Hypotheses and aims

In this sub-study we hypothesised that:

- MR imaging could be used to quantify intra-abdominal fat distribution in pregnant women.
- Improving insulin sensitivity with metformin would result in less deposition of lipid in the more insulin sensitive sites (i.e. visceral, hepatic and skeletal muscle).
- MR imaging could be used to quantify fetal liver volume and fetal subcutaneous fat and hepatic lipid deposition.
- Fetuses of mothers exposed to metformin in pregnancy would accumulate less excess fat.

The aims of the sub-study were to:

- Quantify and compare fat distribution in obese pregnant women exposed to either placebo or metformin in early and late third trimester.
- Quantify and compare hepatic and skeletal muscle lipid in obese pregnant women exposed to either placebo or metformin in early and late third trimester.
- Optimise an imaging protocol for measurement of fetal liver volume, fetal subcutaneous fat and fetal hepatic lipid deposition.
- Quantify and compare fetal liver volume, fetal subcutaneous fat and fetal hepatic lipid in fetuses of obese women exposed to metformin or placebo during pregnancy.

5.3. Methods

5.3.1. Patient recruitment, exclusion and inclusion criteria

All women participating the EMPOWaR trial at the Edinburgh site were invited to attend for an MRI scan at 28 and 36 weeks' gestation. Women with an absolute contraindication to MRI or severe claustrophobia were excluded. Informed consent was obtained at the baseline visit at the time of recruitment to the trial, and reconfirmed at the relevant gestations. All investigations and analyses were performed blind to treatment allocation.

5.3.2. Scan protocol

Whole body MRI and 1H-MRS studies were performed on a Siemens Magnetom Vario 3 Tesla system (Siemens AG, Healthcare Sector, Erlangen, Germany) at the Clinical Research Imaging Centre (CRIC), Queen's Medical Research Institute, Edinburgh.

Participants were positioned in the magnet in a full left lateral position to avoid aorto-caval compression. Data from the abdomen and thigh were acquired using a combination of spine and body matrix coil elements. Aural protection was provided by use of earplugs and headphones. Contact between the participant and scanning staff was maintained at all times. Heart rate and oxygen saturations were monitored continuously throughout the scan period; blood pressure was measured at the start of the scan and every 10 minutes throughout the procedure.

Scans were performed by the CRIC radiographers, led by Annette Cooper (senior MR radiographer). Image analysis was carried out by the investigator (CC). Dr Calum Gray (physicist) carried out the spectroscopy analysis and post-processing work. Dr Scott Semple (MR physicist) developed and optimised the imaging protocols and over saw all data acquisition. He also carried out the image analysis as

a second observer for the inter-rater reproducibility data. All scans were reviewed for clinical reporting by Dr Jane Walker (consultant radiologist).

5.3.3. Scan sequences

Standard localising sequences were acquired to confirm organ and fetal position. MRI and MRS were acquired with a combination of body matrix and spine matrix elements. Two-dimension multi-slice fast low angle shot (FLASH) images were acquired axially, central to the right lobe of the maternal liver with water and lipid signals in and out of phase. MRI and MRS were also acquired in maternal right quadriceps muscle.

5.3.3.1.   Maternal measurements

Quantification of maternal abdominal adipose tissue

To quantify maternal subcutaneous and visceral fat, a 3D T1 weighted volumetric interpolated breath-hold examination (VIBE) sequence was acquired axially through the liver. Lipid signals were defined using a semi-quantitative thresholding technique using the commercial software SliceOmatic^{TM} (TomoVision, Quebec, Canada).

Adipose tissue appears bright on T1 weighted images. Regions of interest with attenuation above an investigator-defined threshold were coloured to define visceral adipose tissue and subcutaneous adipose tissue (Figure 10) Intra-abdominal adipose was defined as all adipose tissue below the abdominal wall musculature. This included omental, mesenteric and paranephric deposits but did not distinguish between intra- and retro-peritoneal fat. Subcutaneous deposits included all adipose inferior to the skin but outwith the abdominal cavity. Breast tissue was excluded from analysis. Adipose tissue volumes were calculated using the extracted areas of the regions of interest (mm^2) multiplied by the width of each slice (2mm) to give a unit of volume in mm^3. This was then expressed expressed as a percentage of the total abdominal volume of the region being examined.

A multi-slice approach was used. The left renal pelvis was identified in all subjects and adipose tissue was measured in 20 x 2mm slices cranial to this level. The renal pelvis was used as a landmark as it was within the region of interest, easily identifiable in all subjects, and not likely to be subject to a significant degree of movement with advancing gestation.
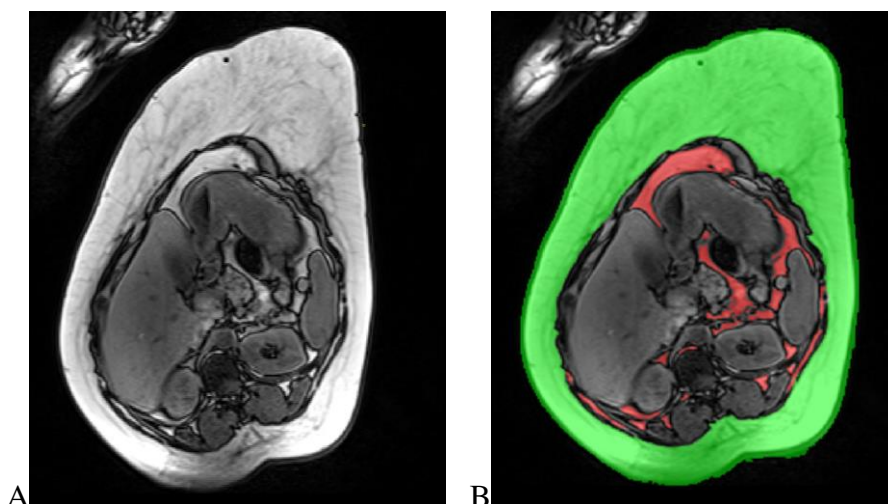

Figure 10 Maternal adipose tissue MRI images
Uncoloured (A) and coloured (B) axial slices to show subcutaneous (green) and visceral (red) fat distribution

Quantification of maternal intrahepatic and skeletal muscle lipid concentration

For ^{1}H-MRS measurement of intramyocellular and intrahepatocellular lipid, single voxel spectra localised to the right quadriceps muscle and the right lobe of the liver were acquired using a point resolved sprectroscopy (PRESS) sequence (TR 5000 ms/ TE 30 ms) with and without water suppression and with 8 signal averages. The voxel size was $2cm^3$ in the muscle and $3cm^3$ in the liver. The voxel site was chosen to avoid large blood vessels or subcutaneous adipose tissue. Lipid concentration was calculated from the water-suppressed acquisition using the spectroscopy analysis tool jMRUI (MRUI Consortium, Brno, Czech Rebuplic).

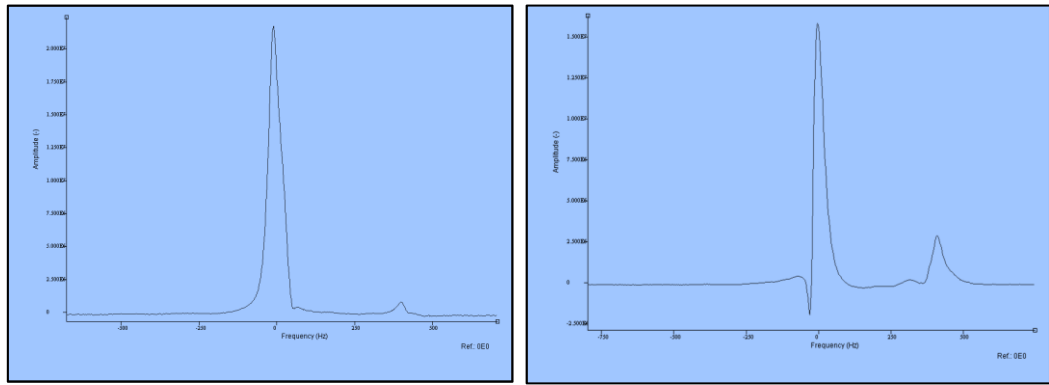Examples of spectra obtained are shown in (Figure 11).

Figure 11 Representative MR spectra from liver (left) and skeletal muscle (right)

In addition to MRS, in- and out-of-phase imaging was used to calculate hepatic and skeletal muscle fat fraction. This is also known as the Dixon method. The lipid signal was calculated by subtraction of in and out of phase images (2.46ms and 8.61ms), and T2* decay during this time corrected using the two in-phase images (2.46ms and 4.92ms) according to protocols which are well established for use in adult liver.[189-191]

5.3.3.2. *Fetal measurements*

Estimation of fetal liver volume

A T2 half-fourier acquisition single-shot turbo spin echo (HASTE) sequence was acquired of the fetus to cover the fetal liver in the axial, sagittal and coronal planes (dependent on degree of fetal movement during the acquisition period).

The fetal liver is identifiable by the investigator on these images using standard anatomical landmarks. The entire liver was coloured as the area of interest on every slice it was visible using the same software as was used for the maternal fat measurements (Figure 12). The area of the coloured region was extracted and converted into a unit of volume (mm^3) by multiplying the area (mm^2) by the width of each slice (2mm).
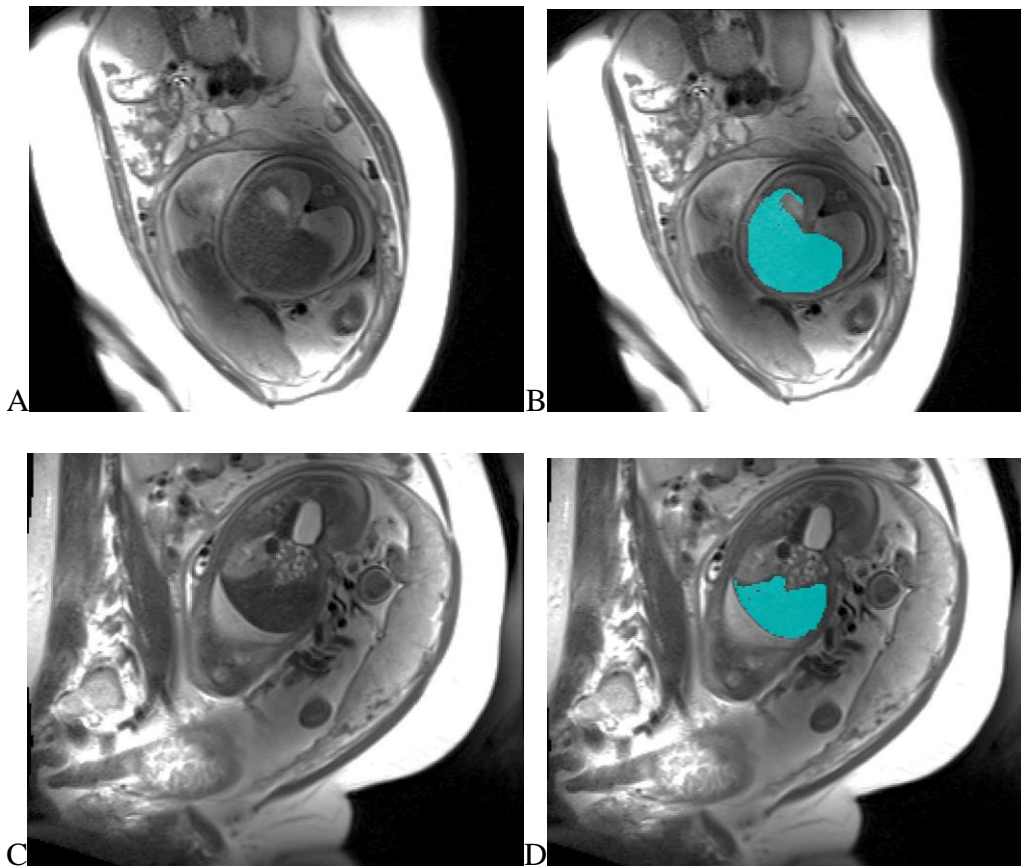
Figure 12 Fetal liver MRI images

Uncoloured (A and C) and coloured (B and D) axial (A and B) and sagittal (C and D) slices to show the fetal liver.

Estimation of fetal hepatic fat

A T1 weighted fast low angle shot (FLASH) sequence was acquired for the in- and out-of-phase fetal liver fat fraction. The slice thickness was 8mm. The lipid signal was calculated by subtraction of in and out of phase images (2.46ms and 8.61ms), and T2* decay during this time corrected using the two in-phase images (2.46ms and 4.92ms) according to protocols which are well established for use in adult liver[189-191].

Estimation of fetal subcutaneous fat

For the fetal subcutaneous fat, a fat excitation FLASH sequence was used, again with 8mm slice width. Shoulder to shoulder coverage of the fetus was obtained in the

sagittal plane and a single slice at the level of the umbilical cord insertion was used in the axial plane. The subcutaneous fat was coloured on every available slice in the sagittal plane (Figure 13) and on the single slice in the axial plane using the same technique as described for the maternal subcutaneous and visceral fat and fetal liver volume. The amount of fat was expressed as a percentage of the total volume of the area examined. These protocols were subject to method development during the study process.
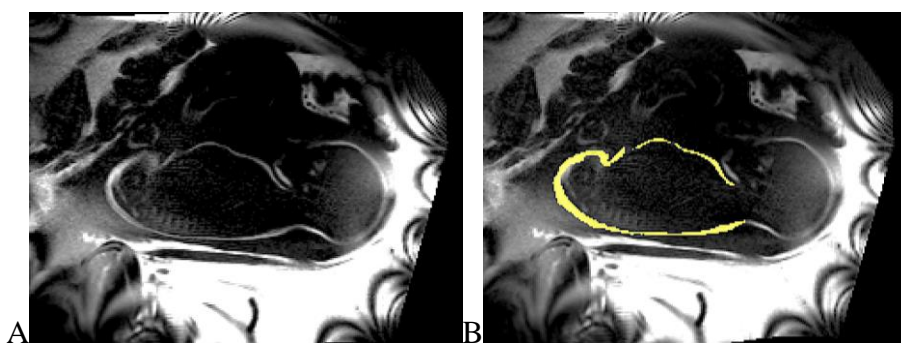

Figure 13 Fetal subcutaneous fat MRI images
Uncoloured (A) and coloured (B) sagittal slices through the fetus to show the subcutaneous fat.

5.3.4. Statistical analysis

Comparisons between groups were made using unpaired t test or Mann Whitney test where data were not normally distributed. Comparisons within groups were made using paired t test, or Wilcoxon test where data were not normally distributed. Kruskal Wallis was used to compare differences between the groups over the two time points. Significance was set at $p<0.05$.

5.3.5. Reproducibility

The intra-observer and inter-observer variability for measurement of the maternal abdominal adipose tissue using this same method has previously been validated and found to be highly correlated[192]. Intra-observer variability for measurements of fetal liver volume and fetal subcutaneous fat were assessed by the same observer (CC)

defining the region of interest on all relevant slices from five subjects on two separate occasions. Inter-observer variability was assessed by comparison of data from all relevant slices for five subjects by two independent observers (CC and SS).

For each paired data set, a correlative plot of the data sets around the line of equality is presented. Agreement was assessed by construction of Bland Altman plots with 95% limits of agreement as follows: upper 95% limit of agreement = mean difference + 2SD; lower 95% limit of agreement = mean difference - 2SD.

5.3.5.1. *Fetal liver volume reproducibility*

Following repeated measures by the same investigator (intra-rater) and of the same images by two investigators (inter-rater), measurements of the fetal liver volume were found to be well correlated in both the axial (Figure 14, Figure 14 Axial fetal liver volume intra-rater reproducibility and Figure 18) and sagittal (Figure 16, Figure 16 Sagittal fetal liver volume intra-rater reproducibility and Figure 19) planes with the majority of points scattered evenly around the line of no difference and within the upper and lower 95% limits of agreement. Measurement in the axial plane shows the best reproducibility. This may be due to the fact that there is less rotation of the fetus on this axis and more reliable images were obtained.
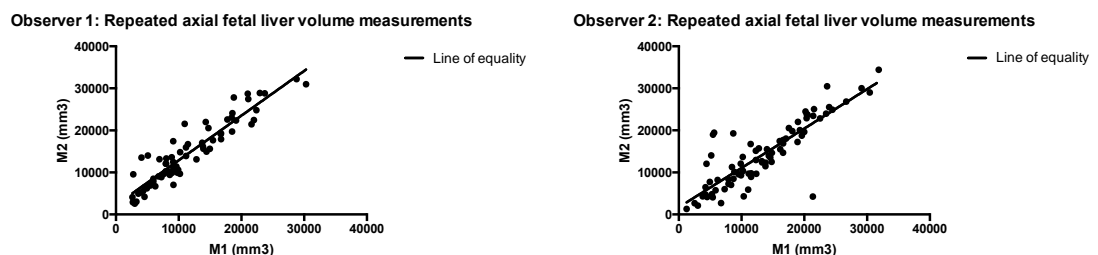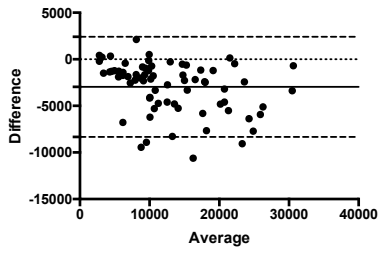
Intra-rater variability in the axial plane

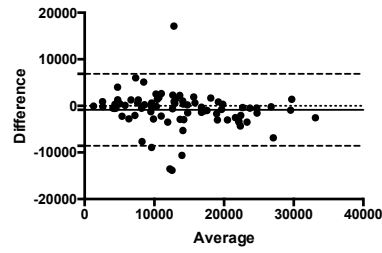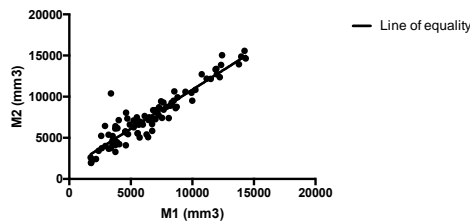**Figure 14 Axial fetal liver volume intra-rater reproducibility**

Figure 15 Axial fetal liver volume intra-rater reproducibility: Bland Altman analysis

Observer 1: n=71 pairs, mean difference (lower – upper 95% limits of agreement) -2965 (-8335 to 2404) mm^3

Observer 2: n=80 pairs, mean difference (lower – upper 95% limits of agreement) -852.9 (-8575 to 6869) mm^3

Intra-rater variability in the sagittal plane
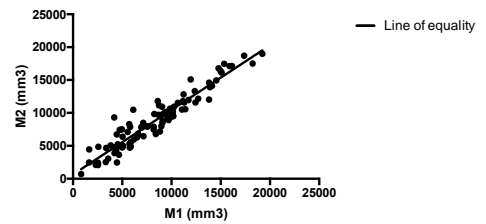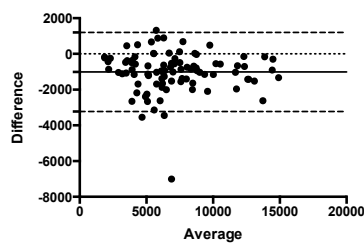
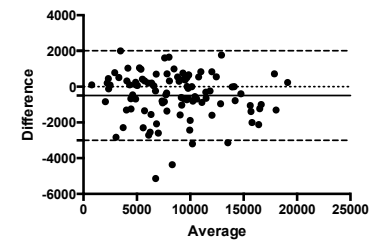**Figure 16 Sagittal fetal liver volume intra-rater reproducibility**

Figure 17 Sagittal fetal liver volume intra-rater reproducibility: Bland Altman analysis

Observer 1: n=93 pairs, mean difference (lower-upper 95% limits of agreement) -1015 (-3228 to 1198) mm^3

Observer 2: n=99 pairs, mean difference (lower-upper 95% limits of agreement) -495 (-3003 to 2014) mm^3

Inter-rater variability in the axial plane

Figure 18 Axial fetal liver volume inter-rater reproducibility

Bland Altman analysis

n=77 pairs, mean difference (lower-upper 95% limits of agreement) -92.84 (-8255 to 8069) mm³

Inter-rater variability in the sagittal plane

Figure 19 Sagittal fetal liver volume inter-rater reproducibility

Bland Altman analysis

n=96 pairs, mean difference (lower-upper 95% limits of agreement) -1110 (4284 to 2065) mm³

5.3.5.2.   *Fetal subcutaneous fat*

Intra-rater variability

Repeated measures by the same investigator were performed. Repeated measures for this parameter were less highly correlated, as demonstrated by a wider scatter of points around the line of equality and between the 95% limits of agreement (Figure 20). This is likely to reflect both the smaller number of suitable images for analysis

of fetal subcutaneous fat and the fact that this was a novel technique that required significant method development during the study period.
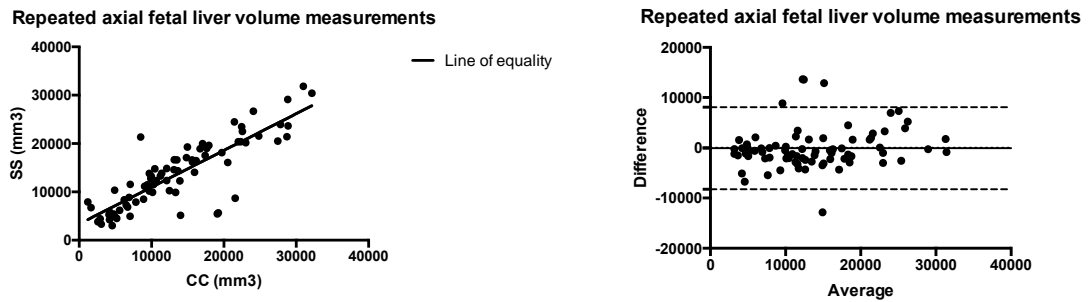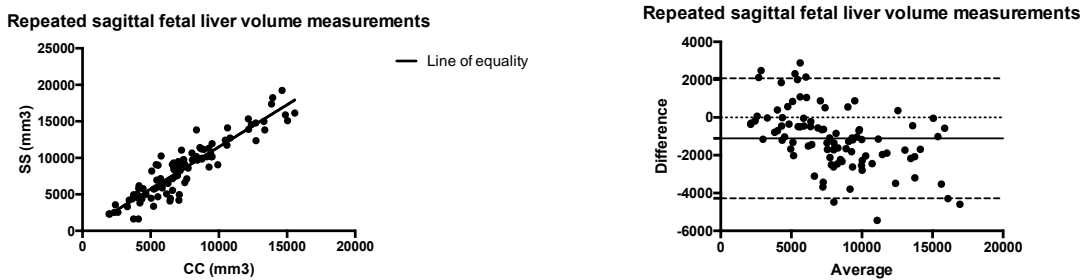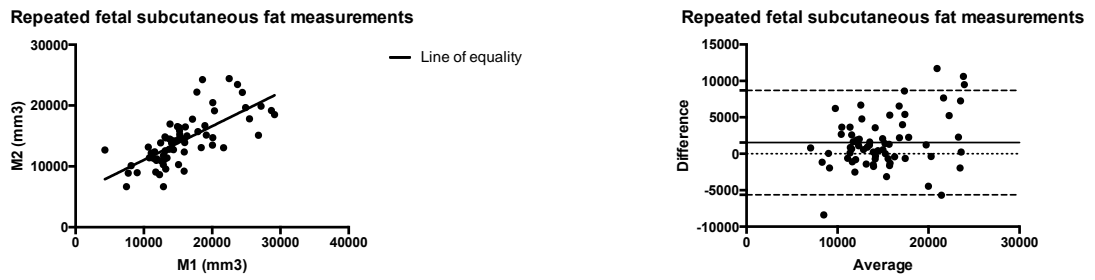

Figure 20 Fetal subcutaneous fat intra-rater reproducibility

Bland Altman analysis n=67 pairs, mean difference (lower-upper 95% limits of agreement) 1530 (-5630 to 8691) mm³

5.4. Results

Demographic characteristics of the participants are shown in Tables 12 and 13.

There is an inevitable unavoidable selection bias in recruitment to the sub-study as it is likely that the more motivated participants agreed to undergo the extra tests. However, there were no significant differences in baseline demographic characteristics between the sub-study population and the trial group as whole (see Table 12). Importantly BMI distribution was the same as the main trial group. All participants in the sub-study were also in the per-protocol analysis group in the main trial, i.e. they fulfilled the pre-defined criteria for compliance with the study treatment.

37 participants (n=18 and 19 in placebo and metformin groups respectively) underwent MRI and ^{1}H-MRS studies at both 28 and 36 weeks' gestation. A further 10 participants (n=6 and 4 in placebo and metformin groups respectively) underwent MRI at 28 weeks' gestation only and 10 participants (n=6 and 4 in placebo and metformin groups respectively) at 36 weeks' gestation only.

	Placebo		Metformin		Placebo ITT		Metformin ITT		Placebo PP		Metformin PP	
	n=30		n=27		(n)		(n)		(n)		(n)	
	Mean or n	SD or %	Mean or n	SD or %	Mean or n	SD or %	Mean or n	SD or %	Mean or n	SD or %	Mean or n	SD or %
Age (years)	29.4	4.5	30.1	5.5	28.9	5.1	28.7	5.8	29.6	4.9	29.6	5.6
Nulliparity	11/30	36.7%	11/27	40.7%	84/223	37.7%	100/226	44.2%	59/155	38.1%	66/139	47.5%
BMI at baseline (kg/m^2)	38.2	5.6	39.4	4.7	37.7 (223)	5.6	37.8 (226)	4.9	37.5 (155)	5.4	37.8 (139)	4.9

Table 12 Demographic characteristics of the participants in MR sub-study.

Data are presented as mean + /-SD or %. Data are show for the participants in the whole trial population for comparison (ITT – intention to treat population, PP – per protocol population). There were no significant differences between the characteristics of the sub-study population compared to either the ITT or PP populations.

	Placebo		Metformin		Placebo ITT		Metformin ITT		Placebo PP		Metformin PP	
	n=30		n=27		(n)		(n)		(n)		(n)	
	Mean or n	SD or %	Mean or n	SD or %	Mean or n	SD or %	Mean or n	SD or %	Mean or n	SD or %	Mean or n	SD or %
Male sex	14/30	46.7%	11/27	40.7%	110/223	49.8%	109/226	50.2%	76/155	49.0%	71/139	51.0%
Birthweight (g)	3493.0	512.4	3596.1	494.7	3463 (220)	659.6	3462 (214)	548	3551.5 (153)	573.6	3498.5 (556.9)	138
Birthweight centile	51.7	29.6	63.4	25.8	57.3 (220)	27.9	56.9 (214)	28.7	59.8 (153)	26.9	58.6 (138)	28.2
Ponderal index at birth	3.44	4.6	2.60	0.32	3.01 (145)	3.68	2.67 (131)	0.5	3.19 (106)	4.28	2.64 (95)	0.44
Fat %	12.53	5.7	12.63	4.3	12.08 (22)	5.74	12.86 (21)	4.5	12.7 (18)	5.64	12.8 (20)	4.56
Triceps skinfold at birth (mm)	11.38	18.1	8.30	2.5	14.34 (111)	20.6	16.4 (99)	27.9	14.7 (90)	21.1	15.9 (83)	28.6
Subscapular skinfold at birth (mm)	10.06	13.9	7.11	2.3	13.46 (113)	20.4	15.7 (98)	27.96	13.4 (91)	20.2	14.6 (82)	28.1

Table 13 Demographic characteristics of the babies of the participants in MR sub-study.

Data are presented as mean + /-SD or %. Data are show for the participants in the whole trial population for comparison (ITT – intention to treat population, PP – per protocol population). There were no significant differences between the characteristics of the sub-study population compared to either the ITT or PP populations.

5.4.1. Maternal subcutaneous and visceral fat masses

There was no difference in the subcutaneous fat mass (expressed as a percentage of the abdominal volume examined) between the placebo and metformin groups at 28 weeks' gestation (difference between means -3.45, 95% CI -7.63 to 0.73, p=0.10) or at 36 weeks' gestation (difference between means -3.43, 95% CI -7.63 to 0.76, p=0.11). There was no difference in visceral fat mass (expressed as a percentage of the abdominal volume examined) at 28 weeks' gestation (difference between means -0.02, 95% CI -1.93 to 1.89, p=0.98) or at 36 weeks' gestation (difference between means 0.18, 95% CI -2.17 to 1.82, p=0.86)(Figure 21).

Both groups demonstrated significant loss of subcutaneous fat mass between 28 and 36 weeks' gestation (paired t test: placebo mean of differences -2.14, 95% CI -3.27 to -1.01, p=0.0009; metformin mean of differences -2.15, 95% CI -3.38 to -0.923, p=0.0018). There was no significant difference in the percentage change in subcutaneous fat mass from 28 to 36 weeks' gestation in the placebo group compared to the metformin group (difference between means -0.45, 95% CI -4.71 to 3.82, p=0.83)(Figure 22).

Neither group demonstrated any change in visceral fat mass between 28 and 36 weeks' gestation and thus there was no difference in change in visceral fat mass between 28 and 36 weeks' gestation between the groups (Mann Whitney test, p=0.60)(Figure 22).
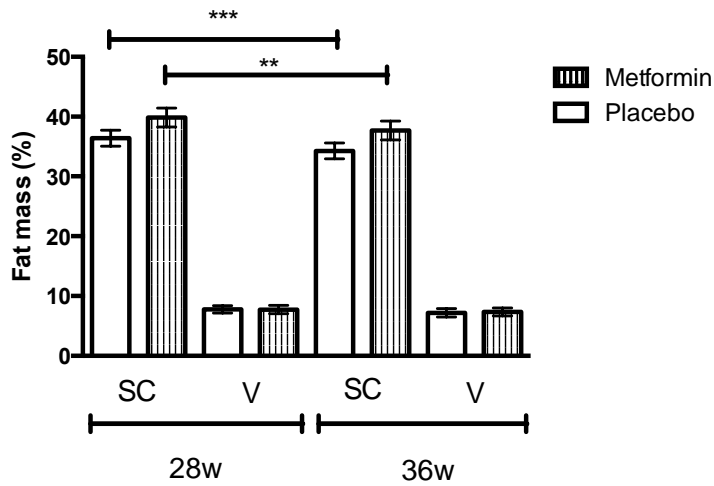
Figure 21 Maternal subcutaneous (SC) and visceral (V) fat mass (%) at 28 and 36 weeks' gestation.

Data are presented as mean +/- SEM. *p<0.05, **p<0.01, ***p<0.0001.
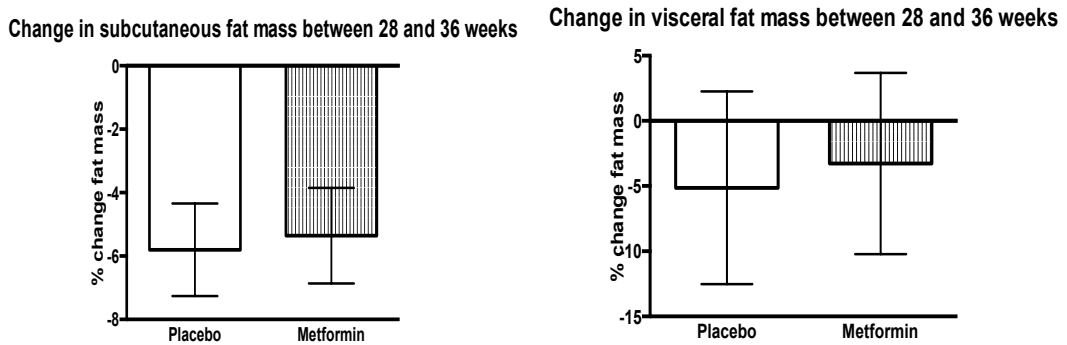
Figure 22 Percentage change in subcutaneous and visceral fat mass between 28 and 36 weeks' gestation.

Data are presented as mean +/- SEM.

5.4.2. Maternal skeletal muscle and hepatic fat fraction

5.4.2.1.   In- and out-of-phase imaging method

The mean (SD) hepatic fat fraction (%) at 28 weeks' gestation was 3.93 (1.35) and 4.90 (2.76) in the placebo and metformin groups respectively. At 36 weeks' gestation it was 5.16 (2.78) and 5.00 (2.54) for the placebo and metformin groups respectively.

The mean (SD) skeletal muscle fat fraction (%) at 28 weeks' gestation was 2.84 (0.90) and 3.20 (1.33) in the placebo and metformin groups respectively. At 36 weeks' gestation it was 3.43 (1.29) and 3.62 (1.82) for the placebo and metformin groups respectively.

There were no significant differences in fat fraction measured by this method by gestation or treatment group in either the skeletal muscle ($p=0.55$) or the liver ($p=0.50$)(Figure 23).

Figure 23 Maternal hepatic and skeletal muscle fat fraction (%) in placebo and metformin groups at 28 and 36 weeks gestation using the Dixon method.
Data are presented as mean +/- SEM.

5.4.2.2. ^{1}H-MRS method

The mean (SD) hepatic fat fraction (%) at 28 weeks' gestation was 1.24 (1.26) and 0.87 (1.22) in the placebo and metformin groups respectively. At 36 weeks' gestation it was 1.11 (1.80) and 0.86 (0.71) for the placebo and metformin groups respectively.

The mean (SD) skeletal muscle fat fraction (%) at 28 weeks' gestation was 7.13 (3.77) and 7.55 (4.86) in the placebo and metformin groups respectively. At 36 weeks' gestation it was 10.99 (9.60) and 9.10 (4.46) for the placebo and metformin groups respectively.

There were no significant differences in fat fraction measured by ^{1}H-MRS by gestation or treatment group in either the skeletal muscle (p=0.64) or the liver (p=0.42)(Figure 24).
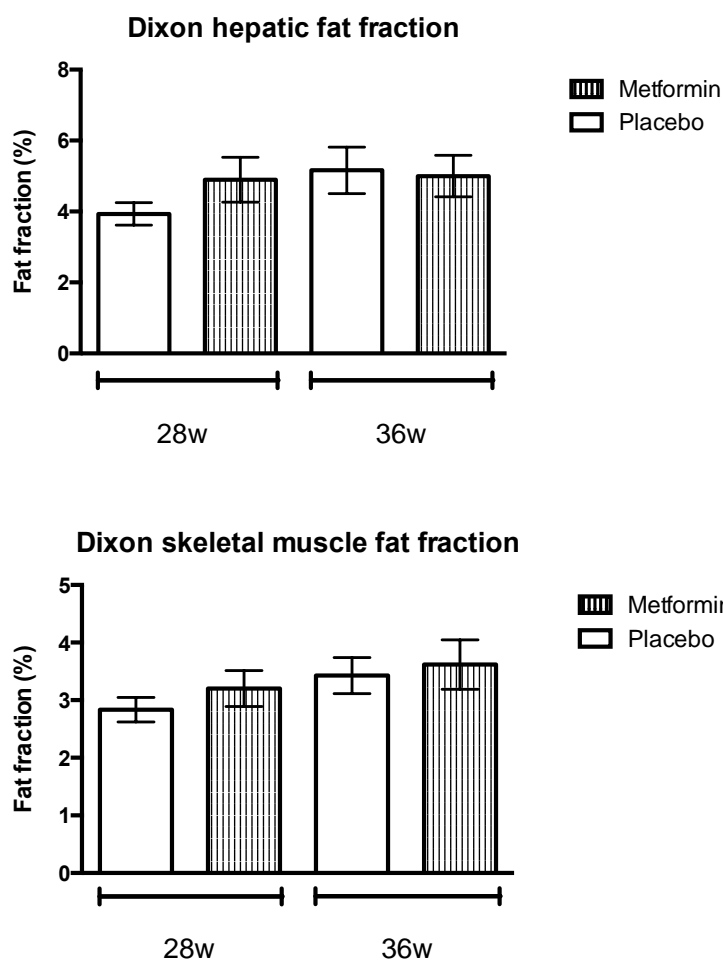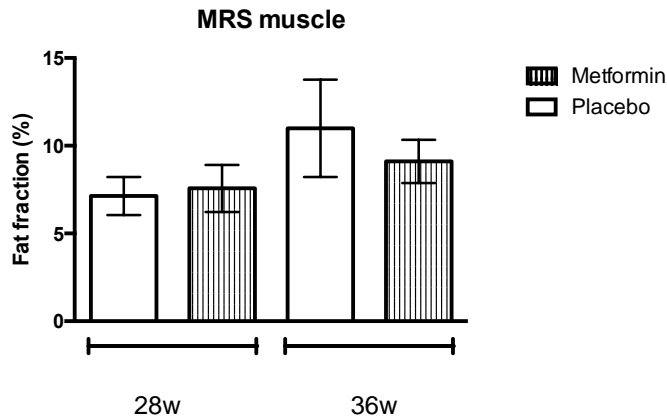
Figure 24 Maternal hepatic and skeletal muscle fat fraction (%) in placebo and metformin groups at 28 and 36 weeks gestation using ^{1}H-MRS.
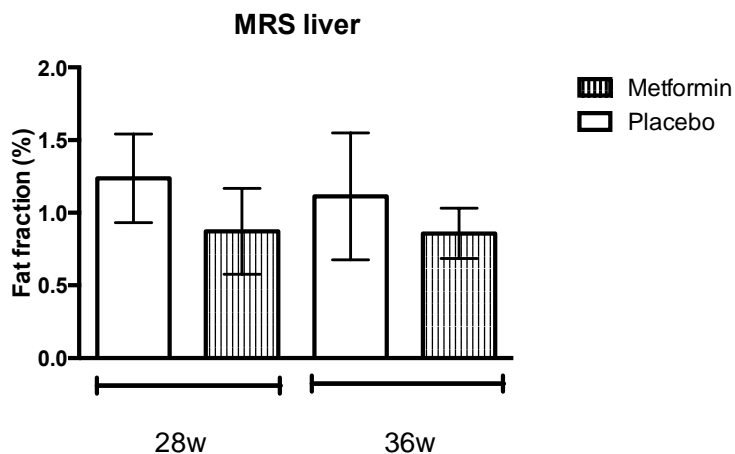
Data are presented as mean +/- SEM.

5.4.3. Fetal liver volume, hepatic fat fraction and subcutaneous fat

There was no statistically significant difference in fetal liver volume at 28 or 36 weeks' gestation between the placebo and metformin treatment groups. Both groups demonstrated a significant increase in liver volume over time ($p<0.0001$), as would be expected, but the percentage increase was not significantly different between the two groups when measured in either plane (Figure 25 and Figure 26). There was no change in the fetal hepatic fat fraction by gestation or treatment group (Figure 27). There was no difference in subcutaneous fat, measured in the sagittal and axial plane (expressed as a percentage of body volume) at 36 weeks' gestation between the two treatment groups (Figure 28).
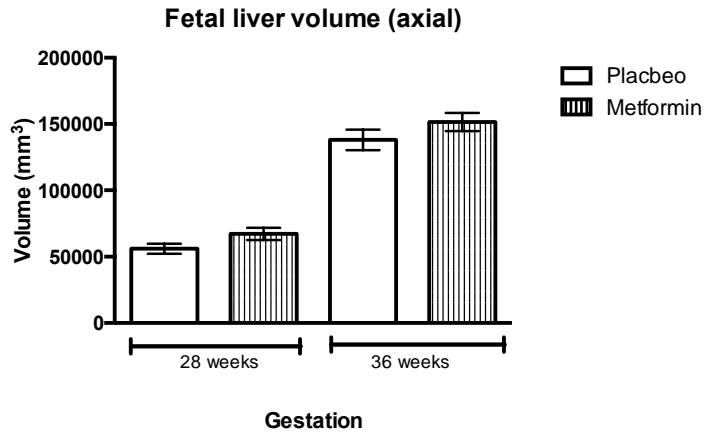
Figure 25 Fetal liver volume (mm³) in the axial plane.

Placebo 28w n=25, 36w n=22; Metformin 28w n=22, 36w n=22. Data re presented as mean +/- SEM.
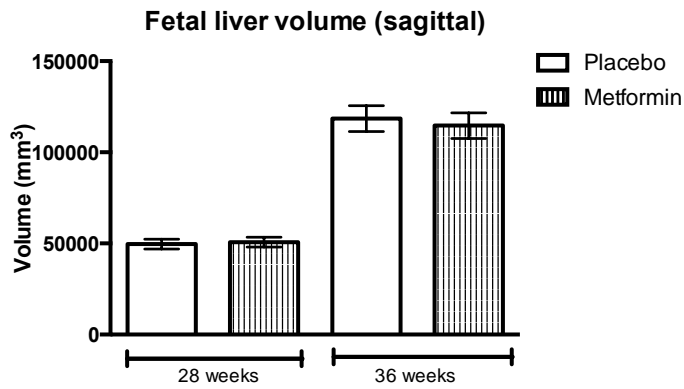
Figure 26 Fetal liver volume (mm³) in the sagittal plane.

Placebo 28w n=25, 36w n=23; Metformin 28w n=23, 36w n=22. Data are presented as mean +/- SEM.

**Figure 27 Fetal hepatic fat fraction (%) measured using in and out of phase imaging.
Placebo n=17, metformin n=16 (paired samples)**

**Figure 28 Fetal subcutaneous fat volume (%) in sagittal and axial planes.
Subcutaneous fat sagittal - placebo n=14, metformin n=25; Axial – placebo n=8, metformin n=5**

5.5. Discussion

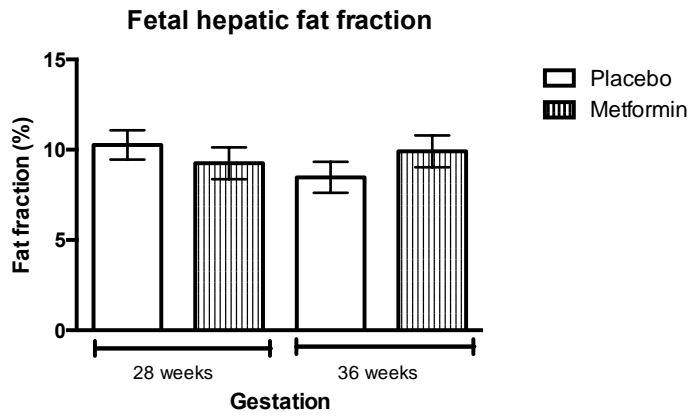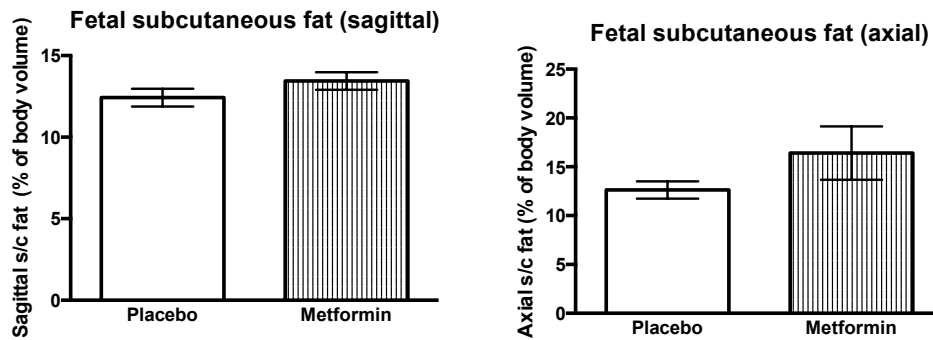The purpose of this sub-study was to assess whether distribution body fat in obese pregnant women was altered by metformin. We also examined the fetus with the aim of assessing effect on liver volume, hepatic fat content and fetal subcutaneous fat deposits. We aimed to scan 40 participants at both 28 and 36 weeks' gestation. Ultimately we scanned 57 participants, with longitudinal maternal data available for 37 participants (those who attended for both scans). We obtained longitudinal fetal hepatic lipid data in 17 and 16 of the participants allocated to placebo and metformin respectively. Liver volume was successfully measured in the axial plane in 25 and 22 fetuses at 28 and 36 weeks respectively in participants allocated to placebo and in 22 subjects at both time-points allocated to metformin. Liver volume was successfully measured in the sagittal plane in 25 and 23 fetuses at 28 and 36 weeks respectively in participants allocated to placebo and in 22 metformin subjects at both time-points. Subcutaneous fat mass was measured in the sagittal plane in 14 and 25 subjects allocated to placebo and metformin respectively. In the axial plane it was successfully measure in 8 and 5 subjects in the placebo and metformin groups respectively.

We have demonstrated that the maternal scanning protocols work well in obese pregnant women, with good inter- and intra-rater correlation. We have shown it is possible to obtain the fetal data as we had planned, though not in every subject scanned due to fetal movement and time limitation, with priority being given to acquisition of the maternal data. There was reasonably good inter- and intra-rater correlation for the fetal liver volume data. Correlation was less good for the subcutaneous fat measurements but the cohort numbers were small for this study.

In summary, participants in both placebo and metformin arms of the study lost subcutaneous fat over the course of pregnancy but there was no difference in the percentage change between the two groups. We saw no differences in amounts of visceral fat either between treatment groups or by gestation. Ectopic lipid deposition

in both the liver and skeletal muscle was also the same in both groups and did not change between 28 and 36 weeks' gestation.

Mean hepatic fat fraction in both groups was relatively low, particularly when measured by ^{1}H-MRS. It is not surprising to see a difference in actual values when comparing the two techniques as they are measuring slightly different things. MRS should be more accurate as it is specifically measuring lipid, whereas the Dixon method is measuring 'non-water'. However, MRS is more prone to artefact and in our particular study population practical considerations, e.g. asymmetrical loading of the subject into the scanner and satisfactory voxel placement away from the dilated hepatic blood vessels, limited spectra acquisition.

When measured using the Dixon method, fat fraction was higher than we have previously seen in a cohort of 10 lean and 10 obese non-diabetic pregnant women[192] but still lower than in a cohort of obese non-pregnant women[193] and certainly below the diagnostic threshold for non alcoholic fatty liver disease[194]. Mean skeletal muscle fat fraction measured by ^{1}H-MRS was similar to that in our previous cohort of obese pregnant women[192] and to that of a group of normally glucose tolerant obese (mean BMI 30kg/m^2) non-pregnant women[195]. This suggests that pregnancy itself, rather than metformin, may be exerting a protective effect on the liver, which deters accumulation of lipid in this site.

Several studies have identified the liver as the primary site of action of metformin in the non-pregnant population[143, 196, 197] but there are limited data examining whether hepatic insulin sensitisation by metformin is associated with a change in liver fat content. While some studies have demonstrated a reduction in hepatic lipid[198], others have shown a neutral effect despite improvement in hepatic insulin sensitivity[199]. However, there are no studies where metformin has been used in isolation in subjects with obesity only without other co-pathologies such as diabetes or non-alcoholic fatty liver disease.

The fetal data must be interpreted with a greater degree of caution. The scanning protocols are not well established and were subject to some method development during the study period. Fetal movement during the image acquisition period is an extra challenge. We aimed to limit the scan duration to 60 minutes, which was the limit of acceptability for the participants, and we prioritised the acquisition of the maternal data in this time. However, we still acquired a reasonable amount of data suitable for analysis and have not demonstrated any differences in the fetal liver volumes or hepatic and subcutaneous fat depots between the placebo and metformin groups. This is in keeping with the primary outcome of the EMPOWaR trial, which demonstrated no difference in birthweight of the babies, and also among the secondary outcomes we saw no difference in neonatal fat mass measured by air displacement plethysmography, or between neonatal skin-fold thicknesses. The fetal hepatic fat fraction we measured was higher than the maternal measures by the same technique (Dixon). To our knowledge, ours is the first study to attempt to measure fetal hepatic fat fraction so no data are available for comparison. There are some data to suggest hepatic fat fraction in neonates, measured by ^{1}H-MRS, is positively correlated with maternal BMI[174] so in our cohort of obese subjects it is possible that the fetal hepatic fraction is high for this reason. However, the fetus undergoes a marked period of body fat accretion during the third trimester[200] so it is not possible to directly compare the fetal data with neonatal data.

In conclusion, we have not demonstrated any effect of metformin in obese pregnant women on maternal or fetal body fat distribution at 28 and 36 weeks' gestation.

6. Chapter 6   Effect of metformin on endothelial function

6.1. Background

Hypertensive disorders are more prevalent in obese populations[201] including those who are pregnant[202]. Obese women are at greater risk of chronic hypertension, pregnancy-induced hypertension and pre-eclampsia[203-206]: for a woman with a BMI > 35 kg/m^2, the risk of pre-eclampsia is twice that of a lean woman[207]. The causal mechanisms behind these associations are not clear but insulin resistance may play a role. Women with diabetes of all types during pregnancy have a heightened risk of pre-eclampsia and women with pre-eclampsia have an increased risk of type 2 diabetes in later life[208]. Other possible contributory mechanisms include endothelial dysfunction, inflammation, dyslipidaemia and oxidative stress: all of which are associated with both obesity and pre-eclampsia[104, 209, 210].

The vascular endothelium is the layer of endothelial cells between the blood vessel wall and the blood stream. It is a key regulator of vascular homeostasis, acting not only as a barrier but also as an active signal transducer for circulating influences that modify the vessel wall tone and phenotype[211]. Endothelial dysfunction is characterised by a shift of the actions of the endothelium towards reduced vasodilatation, and a more pro-inflammatory and pro-thrombotic state[212]. Nitric oxide (NO) is a key endoethlium-derived molecule essential for vascular relaxation[213]. Mechanisms that participate in the reduced vasodilatory responses include reduced nitric oxide bioavailability and oxidative stress[212]. Endothelial dysfunction is associated with most forms of cardiovascular disease, such as hypertension, coronary artery disease, diabetes, and chronic renal failure, and often precedes their clinical manifestations[214]. It has also been demonstrated in disease states in the absence of overt cardiovascular complications, such as the metabolic syndrome [215] and obesity[216].

Endothelial dysfunction in pregnancy has been most widely studied in the context of pre-eclampsia where impaired maternal vascular function has been reported[217]. Gestational diabetes is associated with increased oxidative stress and overexpression of inflammatory cytokines, both of which contribute to endothelial dysfunction[218]. Obesity in pregnancy shares these features and impaired endothelial function has been demonstrated in obese pregnant women[104, 158]. Given that insulin resistance and hyperglycaemia are linked to inflammation, vascular dysfunction and hypertension, these processes are potential mediators for these upstream causative pathways linking endothelial dysfunction with cardiovascular disease in obese pregnant women.

Metformin might be the ideal agent to address and to reverse these abnormalities in obese pregnant women. In addition to its primary function as an insulin sensitising agent, metformin has beneficial effects on the vascular endothelium, lipid profile and oxidative stress[98, 219]. These effects appear independent of metformin's glucose lowering and insulin sensitising effects. Metformin also improves vascular function in a variety of clinical syndromes associated with insulin resistance, for example reducing cardiovascular disease risk in patients with type 2 diabetes[220], and improving endothelial function in patients with type 1 diabetes, metabolic syndrome and polycystic ovary syndrome[145, 219, 221-223].

6.1.1. Assessing endothelial function

Several different methods of assessment of endothelial function have been developed over the past few decades[224]. Currently these methods are restricted to use in the research context and none have as yet been incorporated into clinical practice. The various techniques available for the assessment of endothelial function rely on the basic principle that healthy arteries dilate in response to physical, physiological or pharmacological provocation of the endothelium to release NO and/or other endothelium derived vasoactive substances and then measure the response of the blood vessel being studied. In disease states, the endothelium-dependent dilatation is diminished or absent. However, the vascular response is not only determined by the

function of the endothelium, but also but the structural condition of the arteries. Therefore, to differentiate between the endothelium-dependent and endothelium-independent response, an exogenous NO donor (e.g. glycerol trinitrate) must also be used.

Early techniques employed the use of coronary angiography and intracoronary infusion of acetylcholine with the vascular response begin measure by quantitative angiography[225]. Subsequently less invasive techniques have been developed using predominantly the forearm circulation as a surrogate for the coronary arteries[226, 227] which are more suited to patients who do not otherwise require a coronary angiogram for clinical reasons.

6.1.1.1. *Forearm plethysmography*

This is a semi-invasive technique that measures change in forearm blood flow by venous plethysmography in both arms, before and after infusion of vasoactive substances (e.g. GTN, acetylcholine) into a cannulated brachial artery[226]. Its main advantage is that the endothelium-dependent and independent response can be quantified in a dose dependent manner. It is useful for studying differences in blood flow to various stimuli in the same individual. However, comparison between groups are of limited value due to baseline differences in forearm size, arterial pressure, blood flow and other factors[228].

6.1.1.2. *Finger plethysmography*

The EndoPAT™ (Itamar Medical) device records endothelium-mediated changes in the digital pulse waveform using a pair of plethysmographic probes on the index finger of each hand[229]. It works on the principle that an increase in arterial blood volume in the fingertip causes an increase in pulsatile arterial column changes, thus increasing the measured signal. A blood pressure cuff is place on the arm and inflated to suprasystolic pressure for five minutes to induce reactive hyperaemia. The

contralateral arm acts as an internal control and an index between the two arms is calculated by the equipment software. It is totally non-operator dependent and the equipment is much cheaper than that required for other techniques. Its limitation is that augmentation of the pulse amplitude after reactive hyperaemia is not solely dependent on NO but is also influenced by changes in flow and digital microvessel dilatation (i.e. non endothelial factors)[230].

6.1.1.3. *Flow-mediated dilatation*

This technique, first described by Celermajer *et al.*[227], measures the ability of the brachial or radial artery to respond to the release of endothelial NO during reactive hyperaemia following a 5-minute occlusion of the brachial artery with a blood pressure cuff. The diameter of the artery is measured before and after arterial occlusion using ultrasound, a response that has been shown to be mainly NO dependent[231, 232]. Specialist edge detection software is employed to improve accuracy and reproducibility. The endothelial-independent response is then assessed following administration of GTN. There are several technical considerations to bear in mind in order to standardise the use of the technique as numerous factors affect FMD, including temperature, food, drugs and sympathetic stimuli[233]. Participants should fast for 8 to 12 hours before the study and the procedure should be conducted in a quiet, temperature controlled room. Subjects should not exercise, smoke, ingest caffeine or vitamin C for 4 to 6 hours prior to the test. The technique is non-invasive but it is challenging to perform well and requires significant training and practice to optimise technique. Despite these limitations, FMD is considered the gold-standard non-invasive technique for the assessment of endothelial function.

6.2. Hypotheses and aims

In this sub-study we hypothesised that:

- Improving insulin sensitivity in obese pregnant women with metformin would result in improved endothelial function in late pregnancy, compared to those taking placebo.

The aims of the sub-study were to:

- Optimise the technique of FMD for use in obese pregnant women.
- Measure FMD in early and late pregnancy in obese pregnant women participating in the EMPOWaR trial.
- Compare endothelial function in obese pregnant women taking metformin with those taking placebo and with an additional group of lean pregnant controls.

6.3. Methods

6.3.1. Patient recruitment, inclusion and exclusion criteria

We recruited a subset of women participating in the EMPOWaR trial. All women participating in the trial at the Edinburgh centre were invited to take part. Inclusion and exclusion criteria at baseline were the same as those for main trial (see Chapter 2). Additionally, subjects with a pre-existing hypertensive disorder or who were taking any antihypertensive medications or aspirin were excluded. Women with gestational diabetes were excluded. The characteristics of participants in the sub-study were similar to those of the EMPOWaR study overall. We also included a comparator 'control' group of lean pregnant subjects who, with the exception of BMI, were matched for baseline characteristics to the EMPOWaR study population. These participants were recruited from local 'low risk' antenatal booking clinics. Participants in the EMPOWaR trial were assessed at 12-16 weeks' gestation following randomization but prior to commencing study treatment, and at around 36 weeks' gestation whilst receiving trial medication. All measurements were performed blind to treatment allocation. Lean control subjects were assessed at both 12-16 and 36 weeks' gestation.

6.3.2. Endothelial Function

We assessed endothelial function by measuring flow-mediated dilatation in the brachial artery. Subjects were asked to refrain from eating, smoking or consuming alcohol or caffeine in the preceding four hours. Measurements were obtained in a temperature-controlled room with the subject resting in a semi-recumbent position on a bed. Subjects in late pregnancy had a left lateral tilt applied to avoid aorto-caval compression.

Measurements were made using ultrasound imaging (CX50 Ultrasound system with a 7 MHz linear array transducer, Philips Medical Systems, Guildford, UK) of the brachial artery, 2-5 cm above the antecubital fossa. A baseline rest image was acquired for a period of 60 seconds. Arterial occlusion was performed using a

sphygmomanometric cuff applied below the antecubital fossa, inflated to suprasystolic pressure for five minutes and then released to induce hyperaemia. The brachial artery ultrasound image was recorded for 30 seconds before and five minutes after cuff deflation. Images were acquired with electrocardiogram gating, with measurements made in end-diastole, corresponding to the onset of the R wave. To minimize movement, the scan probe was held in place with a probe-retaining device throughout the period of the study. Images were stored digitally and measurements made using edge-detection software (Vascular Research Tools 5, Medical Imaging Applications LLC, www.mia-llc.com)(Figure 29). Results were expressed as change in arterial diameter (D) divided by baseline diameter:

$$FMD\ (\%) = (\frac{D_{peak} - D_{baseline}}{D_{baseline}}) \times 100$$

The peak diameter (D_{peak}) was taken as the mean of the diameter measurements taken five seconds either side of the peak diameter. The baseline diameter ($D_{baseline}$) was taken as the mean of the 60 seconds of baseline recording. We then measured endothelium-independent vasodilatation. After 10 minutes of rest, a second baseline image was obtained for 60 seconds and then a single low dose (25 µg) of sublingual nitroglycerin (GTN) spray was given and measurement recorded for five minutes. Again, the baseline diameter was taken as the mean of the first 60 seconds and the peak diameter was taken as the mean of the measurements five seconds either side of the peak.

All image analysis was observed as it progressed in real time by the investigator. The image analysis edge-detection software allows for manual correction of caliper placement where the image is not sufficiently crisp. If accurate automated or manual caliper placement was not possible due to poor image quality, the subject was excluded from the data analysis. The decision to discard a trace was made by the investigator.
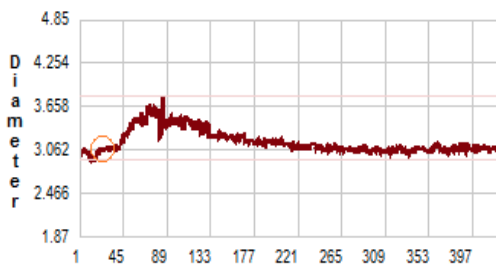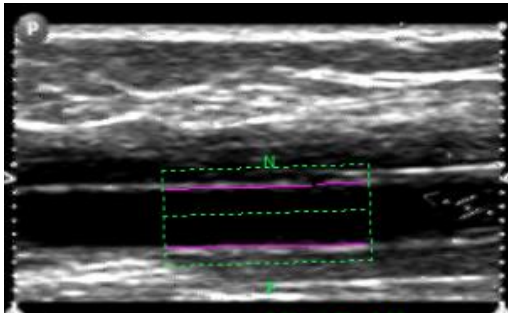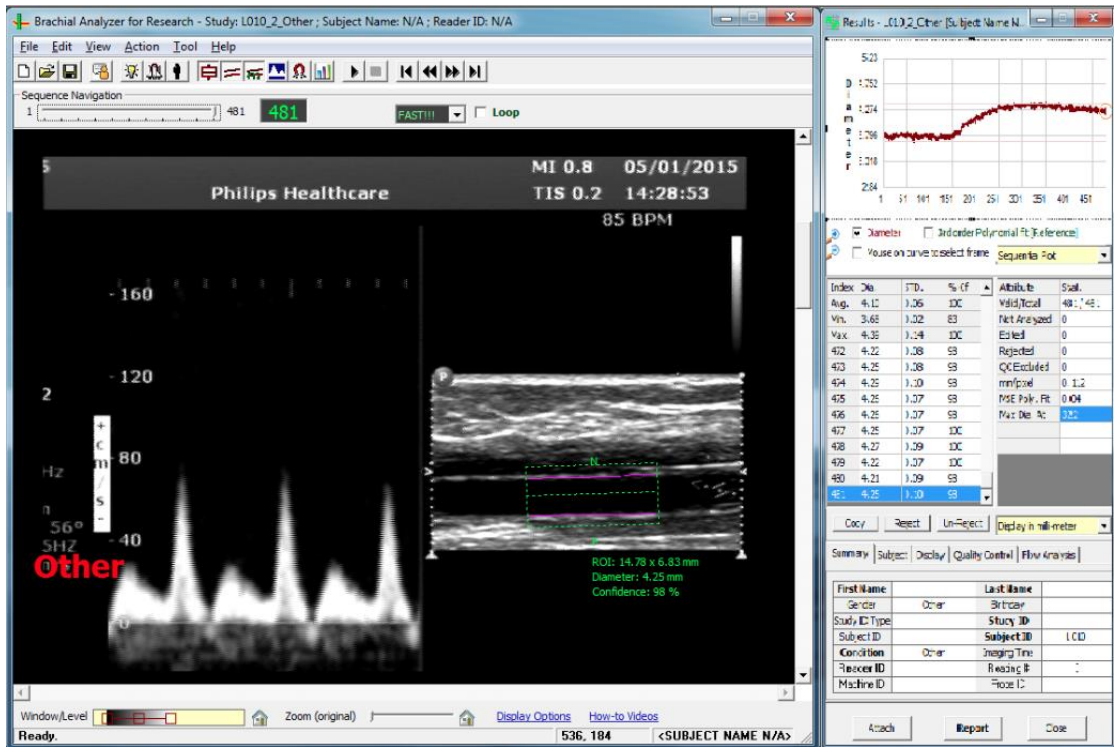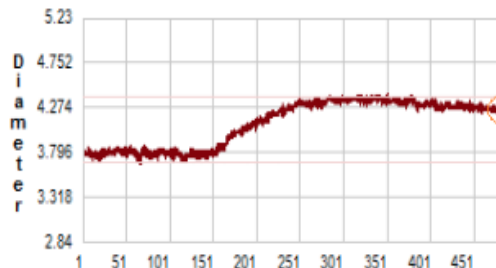
FMD response   GTN response

Figure 29 Representative images from the FMD analysis software

6.3.3. Statistical analysis

Graphical data are presented as mean ± standard error of the mean. Comparisons between placebo, metformin and lean groups at both time points were made using one-way analysis of variance (ANOVA). Two-way ANOVA was used to examine differences over time in the various treatment groups. Comparisons between early and late pregnancy within groups were made using Students unpaired t-test. Significance was taken as a two-sided $p<0.05$.

The candidate carried out all participant recruitment, flow mediated dilatation measurements, analysis of all images and statistical analysis.

6.4. Results

Forty-one eligible women in the EMPOWaR study agreed to participate in the sub-study. However, the majority of EMPOWaR participants were unable or unwilling to attend for the two study visits: only one and two of the placebo and metformin subjects respectively attended both visits. In contrast, all women in the lean control group attended both visits except one lean subject who was ineligible at 36 weeks' gestation due to a pre-term birth. No participants had developed any hypertensive complications of pregnancy or were receiving any medications other than the study tablets. Images from nine subjects had to be discarded as they were of insufficient quality for analysis. The final study population (Table 14) was therefore 28 subjects (6 placebo, 12 metformin and 10 lean) at 12-16 weeks' gestation, and 26 subjects (8 placebo, 9 metformin and 9 lean) at 36 weeks' gestation. All image analysis was carried out prior to unblinding. Following unblinding, all EMPOWaR participants were in the per-protocol analysis group, i.e. they fulfilled the pre-defined eligibility criteria for treatment compliance.

Data are presented for 28 subjects at baseline and 25 subjects at 36 weeks' gestation.

There were no differences in FMD between the placebo, metformin and lean groups at baseline or at 36 weeks' gestation (one-way ANOVA; baseline p=0.88, 36 weeks p=0.89) (Table 15 and Figure 30). There was a decline in endothelial function in late pregnancy compared to early pregnancy across all groups (two-way ANOVA p=0.03). There were no differences in endothelium-independent vasodilatation between groups or within groups in early and late pregnancy (Table 15 and Figure 31).

	Placebo		Metformin		Lean		
	Mean or %	SD	Mean or %	SD	Mean or %	SD	
	n=13		n=19		n=10		*p*-value
Age (years)	31.5	4.4	27.3	6.1	35.6	4.1	
Nulliparity	46.7%		68.8%		70%		
Current smoking	6.7%		18.8%		0%		
BMI at baseline (kg/m^2)	38.4	4.9	37.7	5.7	23.05	3.5	
SBP at baseline (mmHg)	121.7	10.8	117.3	9.7	109.9	15.4	0.06
DBP at baseline (mmHg)	69.1	7.3	69.1	9.5	65.9	10.3	0.63
SBP late pregnancy (mmHg)	123.2	9.1	118.0	15.3	123.8	11.0	0.39
DBP late pregnancy (mmHg)	75.0	6.0	70.4	8.5	73.8	9.6	0.26
Mean gestation at time of study (days)							
Baseline	100.2	10.5	105.2	7.7	100.3	7.7	
Late pregnancy	252.1	4.7	253.3	6.8	257.0	5.6	

Table 14 Demographic characteristics of participants in endothelial function substudy.

Data are presented as mean +/- SD or %.

	Placebo		Metformin		Lean		
	Mean	SD	Mean	SD	Mean	SD	
	n=13		n=19		n=10		*p*-value
FMD (%)							
Baseline (n)	10.16 (6)	5.0	8.58 (12)	6.9	10.22 (10)	10.6	0.88
Late pregnancy (n)	6.08 (8)	5.3	5.10 (9)	4.0	5.40 (9)	3.0	0.89
GTN-mediated dilatation (%)							
Baseline (n)	12.74 (6)	9.7	9.07 (12)	5.6	11.98 (10)	9.0	0.57
Late pregnancy (n)	11.04 (8)	8.1	9.61 (8)*	4.4	8.50 (9)	4.0	0.66

Table 15 Results of endothelial function substudy.

Data are presented as mean +/- SD. *One GTN image had to be discarded as it was of insufficient quality to analyse.

Figure 30 Flow mediated dilatation at 16 and 36 weeks' gestation.

Data are presented as mean +/- SEM. Participant numbers as follows: 16 weeks placebo n=6, metformin n=12, lean n=10; at 36 weeks placebo n=8, metformin n=9, lean n=9. No significant differences between placebo, metformin and lean groups at 16 or 36 weeks (1-way ANOVA; 16w *p*=0.88, 36w *p*=0.89). There was a decline in FMD between 16 and 36w across all groups (2-way ANOVA; *p*=0.03)
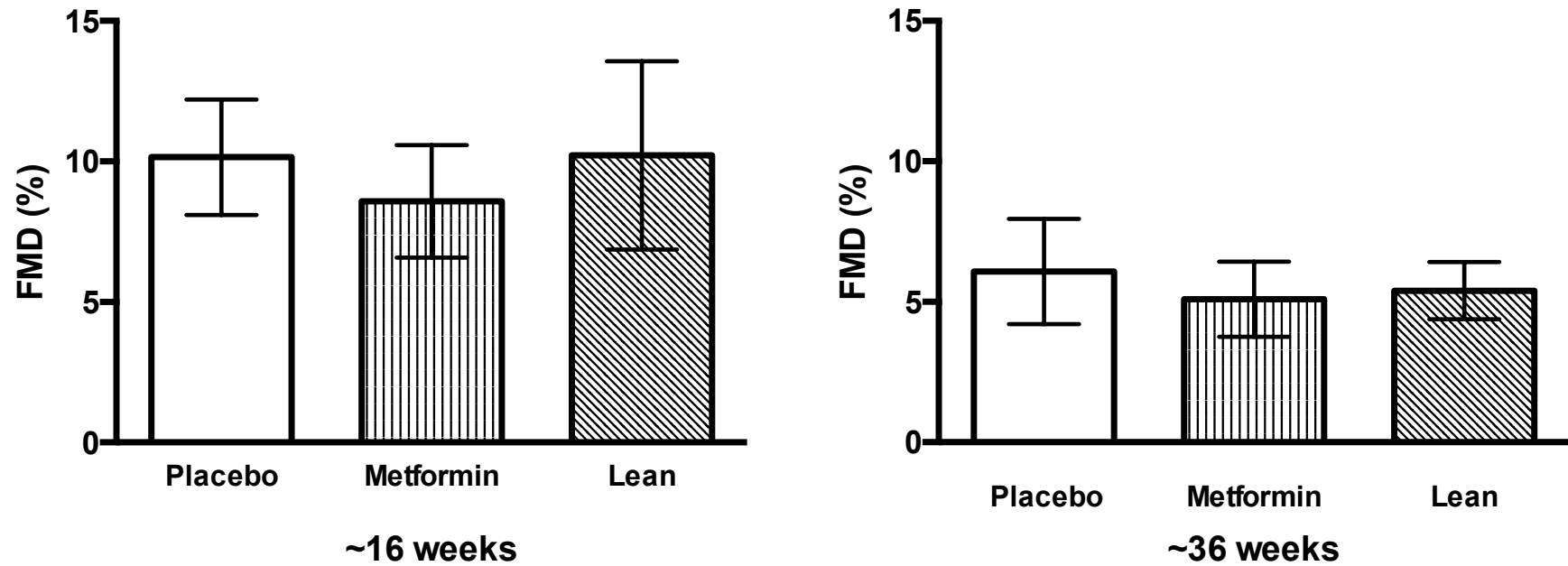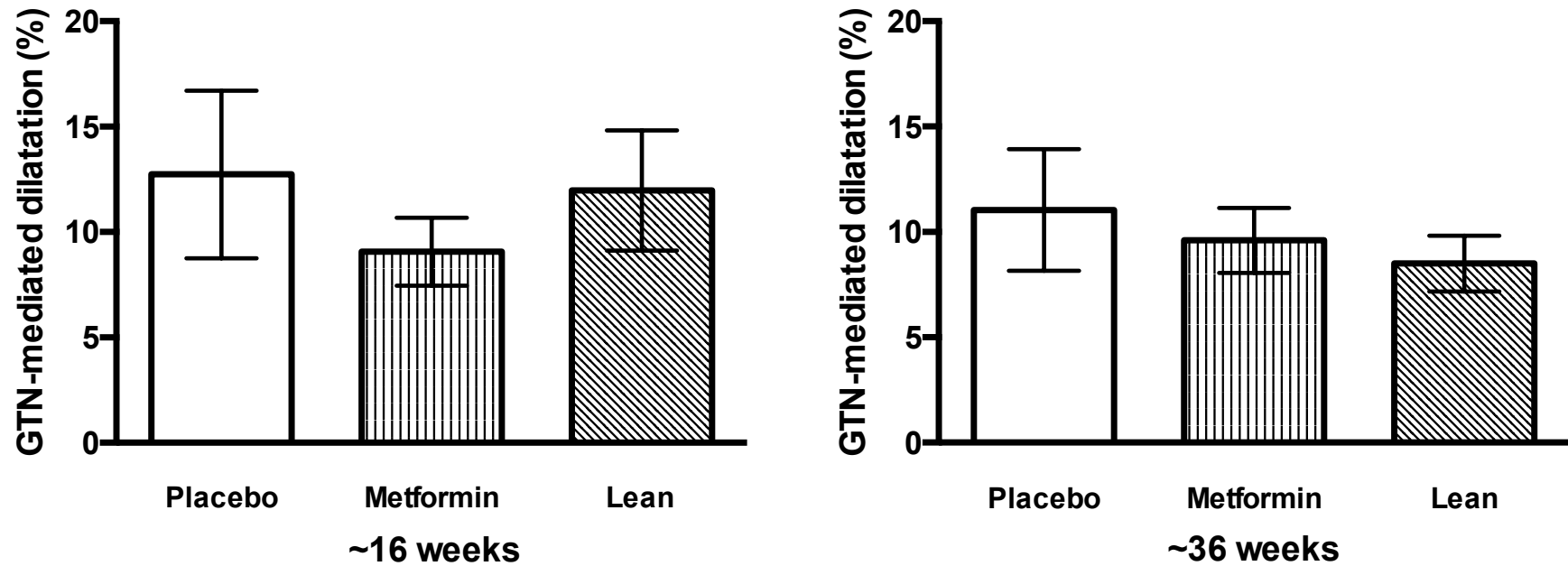
Figure 31 GTN mediated dilatation at 16 and 36 weeks' gestation.

Data are presented as mean +/- SEM. Participant numbers as follows: 16 weeks placebo n=6, metformin n=12, lean n=10; 36 weeks placebo n=8, metformin n=8, lean n=9. No significant differences between placebo, metformin and lean groups at 16 or 36 weeks (1-way ANOVA; 16w *p*=0.57, 36w *p*=0.66)

6.5. Discussion

We have used flow-mediated vasodilatation, the gold-standard non-invasive method of assessing endothelial function, in obese and lean pregnant women as part of the EMPOWaR study. Whilst demonstrating a selective gestation-associated decline in endothelium-dependent vasodilatation, we saw no differences in endothelial function across obese and lean groups, nor any effect of the treatment intervention, metformin. This suggests that pregnancy is associated with altered endothelial function and this is independent of body weight or glucose sensitivity.

There was a decline in endothelial function between early and late pregnancy in participants in all three groups. This is contrary to the finding of one longitudinal study of endothelial function in eight normal weight women who demonstrated increasing FMD across the three trimesters of pregnancy[234]. However, a much larger study of 157 normal weight pregnant women[235] demonstrated an increase in FMD in pregnant compared to non-pregnant subjects, apparent from 10 weeks' gestation but a progressive decline in FMD to pre-pregnancy levels from around 30 weeks' gestation, in keeping with our data in both the lean and obese participants.

To our knowledge, this is the first study of the effect of metformin on endothelial function in pregnant women. It is not clear why metformin does not appear to have had an effect on the vascular endothelium in the study population when this has been demonstrated in other insulin resistant populations. Pregnancy is associated with major vascular and haemodynamic changes, the primary event probably being a fall in peripheral vascular resistance[236]. This is most likely mediated by endothelium-dependent factors including nitric oxide synthesis up regulation by oestradiol and possibly vasodilatory prostaglandins[237-239]. The consequent fall in systemic vascular resistance leads to a compensatory increase in cardiac output. Larger vessels dilate less than smaller ones[227]. It is possible that the increased vessel diameter of pregnancy, along with stimulated nitric oxide activity of pregnancy may obscure any

effect of metformin on the endothelium. However, we did not see any differences in endothelium-independent vasodilatation, which we would expect to see if this was purely a vessel size effect or related to increased nitric oxide consumption. The duration of metformin treatment of 24 weeks may also be a factor in the apparent lack of effect. However, other studies have demonstrated an effect on endothelial function with only a three-month duration of treatment[223, 240].

Flow-mediated dilatation of the brachial artery is accepted as the gold standard for the non-invasive measurement of endothelial function since it is widely used, well-tolerated, low risk and importantly for our study population, suitable for use in pregnant women. There are however some limitations. Most notably for our study population, measurement of the vessel diameter can be technically challenging, particularly in the obese where visualization of the intima is difficult as the ultrasound signal is attenuated but the subcutaneous fat.

A further limitation of this study is the lack of longitudinal data for subjects other than those in the lean group. Having baseline and 36-week data for the same groups of subjects would enhance the statistical power of the findings but this was not possible. This is one of the challenges of performing nested studies within the confines of a larger RCT. Participants often do not have the time or willingness to have multiple extra investigations over and above those required for participation in the 'main' trial. This is perhaps particularly true of a population of young women who already require various appointments for their basic pregnancy care and also often have jobs, children or other demands on their time.

Further exploratory analysis using a linear regression model to examine the association between endothelial function and other continuous variables in addition to metformin such as blood pressure, inflammatory markers (e.g. CRP, IL-6) and HOMA-IR would be interesting but without the longitudinal FMD for each subject it would not be possible to use each participant as her own control.

Other limitations include the small sample size and the variability of baseline demographics (e.g. age, smoking status and parity) within the unblinded groups. This makes it difficult to identify true differences within the small sample size. Clearly an imbalance in treatment group allocation is purely due to chance and unavoidable in the context of a double-blind trial but a larger sample size would help to control for this.

It is possible our sub-study was under-powered. However, we have demonstrated change in FMD over the course of pregnancy (and an absence of change in GTN-mediated dilatation) of a magnitude consistent with other published data. Additionally, in the larger EMPOWaR cohort as a whole, we saw no differences in blood pressure or the incidence of hypertensive disorders of pregnancy, which we may have expected if the placebo group had impaired endothelial function compared to the metformin group. This suggests our findings of no difference between the two groups may be correct rather than a type two error. In conclusion, we have not demonstrated any effect on endothelial function with metformin in obese pregnant women.

7. Chapter 7   General discussion and conclusions

7.1. Summary of findings

In this thesis, we have presented the findings from the first randomised controlled trial of a pharmacological intervention, metformin, to reduce the risk of excessive birthweight in the offspring of non-diabetic obese pregnant women. Contrary to our hypothesis, metformin had no effect on our primary outcome of birthweight. In addition, we did not see any effect on our secondary outcomes of insulin sensitivity at 36 weeks' gestation; maternal and neonatal anthropometry; neonatal CRP, glucose and insulin measured in cord blood.

The inflammatory markers CRP and IL-6 were both lower in the metformin group. These markers are known to be elevated in obese pregnant women compared to lean pregnant women[104] and may be associated with adverse pregnancy outcomes such as pre-term birth and pre-eclampsia[116, 117].

Fasting glucose and insulin were lower in the metformin group at 28 weeks' gestation in the intention to treat analysis. On per-protocol analysis fasting and two-hour glucose, insulin and HOMA-IR score were all lower in the metformin group. The lack of effect at 36 weeks' gestation may reflect the changes in glucose homeostasis throughout pregnancy with the physiological insulin resistance of pregnancy being to overwhelming at this gestation to be influenced by metformin.

The intervention was acceptable to the women who agreed to participate in the trial. No participants were withdrawn specifically because of treatment side effects. Overall adherence was around 60% by both diary entries and detectable levels of metformin in the 36 weeks' gestation blood sample. The median dose taken was 2000g, which suggests the treatment regimen was acceptable to most participants.

The lack of effect overall was evident in both the intention-to-treat and per-protocol analyses. Our study was adequately powered and we can conclude that our results reflect a true lack of effect of the intervention, rather than a type two error.

We also examined the effect of metformin in a series of nested mechanistic substudies. We did not see an effect of metformin on endothelial function, or on maternal and fetal body fat distribution. In subjects undergoing a hyperinsulinaemic euglycaemic clamp study, with concomitant use of stable isotope tracers at 36 weeks' gestation we saw higher endogenous glucose production and insulin stimulated glucose disposal in participants taking metformin, but no differences in whole body glucose disposal or lipid metabolism. Although the numbers of participants taking part in the substudies was small, they were characteristically similar to the study population as a whole and the findings concur with the overall findings in the main trial.

7.2. Strengths and limitations

This was a multicentre study with a double-blind randomised-controlled design making the findings robust and generalisable.

Recruitment to the trial was challenging. The majority of women we approached declined to participate. We were unable to formally assess the reasons for this but anecdotally there was an understandable reluctance among pregnant women to take medication in pregnancy and also a lack of awareness of the potential harm associated with obesity in pregnancy. Although we were formally unable to quantify this, our impression was that women felt stigmatised at being identified as obese and immediately rejected the study on that basis. Additionally, it was difficult to explain to potential participants the risks of maternal obesity and high birthweight for the child. Women did not see having a large baby as an adverse outcome and so the

concept of taking a tablet to prevent this was insufficient to overturn the generic advice not to take medications in pregnancy, unless absolutely necessary.

Despite challenges with recruitment, we were still able to recruit our target sample size and we had adequate power to address our hypothesis.

We have used a recognised surrogate, birthweight centile, as a marker of future life risk of obesity in the offspring. There is good evidence that birth weight is a valid marker to use, from both a systematic review[17] and further supported by large epidemiological studies[15, 16]. A limitation of the study is that follow up is limited to the early postnatal period and longer term conclusions about the effect of metformin on the offspring will require long-term follow-up studies. Indeed, the greatest differences in offspring weight as a result of an intervention in pregnancy may not happen until childhood, as was seen in the 2-year follow-up study[101] of the infants whose mothers participated in the MiG trial[100]. Additionally, the large number of secondary outcomes means that conclusions about these results (even where $p < 0.05$) are potentially subject to a Type I error.

We did not attempt to assess whether blinding was effective. Metformin causes gastrointestinal side effects, so it is possible that some women (and their caregivers) may have correctly inferred their treatment allocation from their side effect profile. However, the majority of the clinical outcomes (e.g. birthweight centile) are unlikely to be significantly affected by observer bias, so we do not think this will have adversely affected the results.

Duration of treatment may have also had an impact. Participants commenced treatment between 12 and 16 weeks' gestation. In studies of metformin use in pregnant women with PCOS, treatment is initiated before or around conception. A longer duration of treatment is associated with greater weight reduction[241]. However, clearly an intervention to be used in pregnancy is limited by the length of the

gestation. It also practically challenging to identify, recruit and randomise women very early in pregnancy before they have registered with antenatal services.

Since publishing the results of the EMPOWaR study[140], another group has published a similar study, with a slightly smaller sample size, and has again found no effect of metformin on birthweight in obese pregnant women without diabetes. The Metformin in Obese Nondiabetic Pregnant Women (MOP) trial[69] was a double-blind, placebo-controlled trial of metformin versus placebo in pregnant women without diabetes who had a BMI greater than 35 kg/m^2. Four hundred and fifty participants were randomised from three UK NHS hospitals, of which 50 withdrew consent following randomisation, leaving 202 in the treatment group and 198 in the placebo group for inclusion in the primary outcome analysis. The primary outcome was neonatal birthweight z score adjusted for gestational age. Secondary maternal outcomes included gestational weight gain, gestational diabetes, pre-eclampsia, pregnancy induced hypertension, delivery by caesarean section, and postpartum haemorrhage. Secondary fetal outcomes included death before 24 weeks' gestation, stillbirth after 24 weeks' gestation, preterm birth, large for gestational age, birth trauma, low Apgar score, hypoglycaemia at birth, hyperbilirubinaemia, and respiratory distress. There were no significant differences between the metformin group and the placebo group in the median neonatal birthweight z score (metformin group 0.05 (IQR -0.71 to 0.92), placebo group 0.17 (IQR -0.62 to 0.98, p=0.66). The authors report significant differences in median gestational weight gain (4.6kg (IQR 1.3 to 7.2) versus 6.3kg (IQR 2.9 to 9.2), p=<0.001) and incidence of pre-eclampsia (3% in metformin group versus 11.3% in placebo group, OR 0.24 (95% CI 0.10 to 0.61) p=0.001) in favour of metformin treatment. There were no significant differences in any of the other maternal or fetal secondary outcomes.

There were some notable differences between the MOP trial and EMPOWaR. Women required a BMI of 35kg/m^2 for entry into MOP, which is higher than the 30kg/m^2 for EMPOWaR. There is a linear relationship between BMI and risk of adverse pregnancy outcome, increasing BMI being associated with increasing risk.

The MOP group used 35kg/m^2 cut-off to enable the study to have adequate power with a smaller sample size. However, they acknowledge the study remains underpowered for any of the secondary outcomes including incidence of pre-eclampsia. It could potentially have made the findings less applicable to the obese population (defined as BMI>30kg/m^2) had they demonstrated benefit of treatment.

The exclusion criteria with regard to diabetes history for participants in the MOP study are not clear. In the pre-published protocol (available at NEJM.org), 'diabetes at booking' is given as an exclusion criterion. The definition of diabetes (e.g. type 1 or 2 or GDM) is not given, and the diagnostic criteria applied are not stated. In the published paper, GDM in a previous pregnancy is listed as an exclusion criterion, but not diabetes at booking. Participants underwent an OGTT at the recruitment visit (12-18 weeks' gestation) but it is not clear whether the results of this test were revealed to the study team or clinical team or whether this occurred before or after randomisation.

In our study, women underwent an OGTT at the baseline visit, prior to randomisation and were excluded if the results met the diagnostic criteria for GDM as per IADPSG, WHO or any locally used criteria. We anticipated that women diagnosed with GDM after enrolment would wish to withdraw and be treated with metformin as a first-line agent. Hence, this baseline test would prevent withdrawals (and protocol violations) by identifying early women who would later be diagnosed with GDM. In practice, this approach may have 'screened out' women most likely to have had had a macrosomic baby and as such most likely to have benefited from treatment. However, this approach allows us to more reliably assess the impact of obesity on pregnancy outcomes rather than hyperglycaemia *per se.*

Whether the MOP trial included or excluded women with GDM, and by which diagnostic criteria, is not known from the groups published material but it is interesting that there was no effect of treatment on the primary outcome regardless of this. They also did not see any difference in the number of women who developed

gestational diabetes at 28 weeks' gestation (although again, no diagnostic criteria are stated).

We used a starting dose of metformin of 500mg, with a maximum allowable daily dose of 2500mg, and up titrated by 500mg per week. In the MOP study, the starting dose was 1000mg, with a maximum dose of 3000mg, and the up titration rate similar. In clinical practice, most clinicians up titrate more rapidly. It is possible that a different dose regime may have produced a different result. Metformin clearance is faster in pregnancy and our study population were obese so a higher dose may have been more effective. The MOP trial researchers chose to use the higher dose to avoid potential criticism, in the event of no effect, that the dose was inadequate. However, the MOP study also did not demonstrate any effect of metformin on the primary outcome of birthweight centile.

In the MOP trial, adherence to treatment was assessed by counting of tablets returned by the participant at each visit. If a patient forgot to return the tablets, verbal report was relied upon. No metrics are given as to how frequently verbal report was relied upon. Adherence was deemed to be good if the total number of tablets consumed was at least 50% of the total prescribed and poor if it was less than 50%. Adherence was good in 82.7% of those taking metformin and 76.3% of those taking placebo, with no significant between-group differences in either adherence or maximum tolerated dose. In EMPOWaR, the maximum tolerated dose in the metformin group was lower than in the placebo group (2000mg compared to 2500mg). We used participant diary records in addition to counting of returned tablets as our assessment of adherence. If no diary or tablets were returned, the participant was assumed to be non-compliant, rather than relying on a verbal report. A further difference is we considered the participant to be compliant if they took at least one tablet on at least 50% of the available tablet-taking days, rather than 50% of the total prescribed. This is a lower dose compared to the MOP study but still compatible with an effective treatment dose. We also examined adherence in the active treatment group using GCMS analysis for presence or absence of metformin in a blood sample taken from the

participants at 36 weeks' gestation. This measure was not used as our primary measure of adherence as no such similar test was available for the placebo group. However, detectable levels of metformin were present in 61% of participants who gave a sample at 36 weeks, which correlates very favourably with the 67% deemed compliant by our pre-defined criteria.

In both MOP and EMPOWaR, significantly more participants in the metformin group experienced gastro-intestinal side effects, which also suggests that those in the active treatment group were taking their treatment.

A further difference between the two studies was ethnicity of recruits. Our study was restricted to white women, primarily to minimise the effect of ethnicity on birthweight. There was no such restriction on recruitment to the MOP study. Over 30% of participants in MOP were of other ethnic groups, with the majority of those being black. There is some evidence to suggest that the efficacy of metformin on glycaemic control is influenced by ethnicity. For example, one study[242] demonstrated a statistically significantly greater impact of metformin on lowering HbA1c in African-Americans compared to European-Americans. The inclusion of all ethnic groups may have made the MOP trial results more generalisable to their local population. However, the potential influence on efficacy must be borne in mind, along with the well-recognised impact of ethnicity on birthweight.

It may be that we did not observe an effect of treatment on our primary outcome because of suboptimal study drug adherence. However, we believe metformin did have its expected pharmacodynamic effect given the differences in measures of insulin sensitivity at 28 weeks' gestation. There was also evidence of a maternal benefit in the active treatment group by a significant reduction in inflammatory markers CRP and IL6. The MOP protocol states that blood samples would also be taken for fasting insulin and inflammatory variables at baseline and 28 weeks' gestation. The group have not yet published any of these results so we do not know whether there was a similar effect of treatment on insulin resistance and

inflammation in mid-gestation that we observed in EMPOWaR but this would be interesting data especially given the reduction in incidence of pre-eclampsia that this study reported.

7.3. Implications for healthcare and recommendations for future research

Obesity is a major public health concern of our time. Rates of obesity among young women of reproductive age are ever increasing and the cycle of disadvantage is thus being perpetuated to the next generation. On the basis of this research, we can conclude that metformin should not be used to improve pregnancy outcomes in obese pregnant women without gestational diabetes. Follow up studies of the babies born to the women who participated in EMPOWaR will be important to determine whether there are any longer term benefits (or indeed harms) of metformin taken during pregnancy.

As previously discussed, despite not seeing an effect on our primary outcome, longer-term effects may become apparent in years to come, such as was observed in the MiG TOFU study[101]. These effects may be beneficial such as a more favourable body fat distribution or cardiometabolic profile for the offspring. However, we must be mindful of the intricate and complex effects the intrauterine environment has on future life risk of health and disease and there is potential that intervention with metformin may have caused harm.

It is biologically plausible that therapy could cause a mismatch in fetal potential and development and thus an adverse adult phenotype. The harmful effects of a mismatch between the intrauterine and extrauterine environment were originally described in relation to lower birth weight and increased rates of cardiorespiratory disease in adulthood[243]. This 'developmental origins of health and disease' hypothesis originated from UK data but has since been replicated worldwide[244]. The original association with birthweight was graded across the normal range of birthweights, not just those born prematurely or with a birthweight low for

gestational age[245-247]. Subsequent animal models have provided clear evidence that the intrauterine environment influences the biology of offspring and that early environmental influence can induce metabolic or endocrine changes in later life[248].

It is possible that the link between early life environment and adult disease may have an evolutionary basis. The 'predictive adaptive responses' hypothesis proposes that there is an evolutionary advantage to an organism to alter its development to optimise its survival in a predicted postnatal environment[249]. This theory suggests long-term consequences may be particularly harmful if there is a mismatch between the 'predicted' postnatal environment and the actual postnatal environment. This has perhaps been most clearly demonstrated in societies where there has been rapid economic or social change, for example in India where there has been a significant population move from a rural, active lifestyle with low calorie diet to a more urban, sedentary lifestyle with a more typically Western diet and consequently a very high prevalence of cardiometabolic disease. Maternal disease, such as obesity, or therapy during pregnancy may similarly cause the fetus to adjust its development inappropriately and thus be less well adapted to cope with a mismatched extrauterine environment. We know offspring of women who are obese in pregnancy are born with existing metabolic compromise; they are more resistance to insulin and have a greater degree of adiposity. We would argue that this perpetuates the cycle of the disadvantage of obesity but offspring may have made this adaptive response to the obesogenic intrauterine environment to prepare for the likely obesogenic extrauterine environment in which they will be raised. Pharmacological intervention during pregnancy may protect the fetus from being born in a metabolically disadvantaged state, which intuitively would seem beneficial but may in fact be harmful if this induces a 'mismatch'. Without long-term follow up studies it is impossible to be certain.

Attention should perhaps instead be focused on lifestyle intervention strategies, although our findings of no beneficial effect are similar to those of other trials of various dietary and lifestyle interventions aimed at reducing birthweight in obese

individuals. However, what is more promising about lifestyle intervention strategies, compared to pharmacotherapy, is that as a consequence of participating in a lifestyle modifying trial, women may be more informed and therefore motivated to maintain a healthier lifestyle and make better food choices in the postnatal period for both themselves and their children.

An alternative approach would be to optimise the diagnosis of GDM in obese pregnant women. Although national recommendations are that obese women should have a glucose tolerance test at 28 weeks to test for GDM, these recommendations are incompletely applied. Additionally, given that glucose levels are high in obese women from the beginning of pregnancy, deferring diagnosis until 28 weeks allows high maternal glucose to impact adversely on fetal growth for the first two thirds of pregnancy. Hence earlier diagnoses for GDM might be appropriate.

In conclusion, antenatal dietary, lifestyle and drug interventions for obese women have thus far not been shown to have a meaningful impact on birth outcomes. It seems the focus of intervention must shift towards reducing weight and optimising health in young girls and women prior to embarking on pregnancy and continuing to prioritise the care of women in the postnatal period to help optimise their health in preparation for subsequent pregnancies. This is unarguably a much greater challenge, which will require broad social change. This must begin with increasing awareness among the general public of the impact of obesity on both immediate and long-term pregnancy outcomes. This heightened public awareness will help to drive the pressing need for the political will to change policies, in line with the recommendations of the United Nations Sustainable Development Goals on the prevention of non-communicable disease.

8. Appendix

The full study report for the National Institute for Health Research Library is included on a CD as an appendix to this thesis.

This has been published by *Efficacy and Mechanism Evaluation, National Institute for Health Research Journals Library* as Efficacy of metformin in pregnant obese women, a randomised controlled trial by **Carolyn A Chiswick**, Rebecca M Reynolds, Fiona C Denison, Amanda J Drake, Shareen Forbes, David E Newby, Brian R Walker, Siobhan Quenby, Susan Wray, Andrew Weeks, Hany Lashen, Aryelly Rodriguez, Gordon D Murray, Sonia Whyte, Ruth Andrew, Natalie Homer, Scott Semple, Calum Gray, Marian C Aldhous, Karen Noble, Sarah Cunningham-Burley, Alice Keely and Jane E Norman.

9. References

1.   Obesity: preventing and managing the global epidemic. Report of a WHO consultation. World Health Organization technical report series. 2000;894:i-xii, 1-253.
2.   World Health Organisation.
3.   Diet, nutrition and the prevention of chronic diseases. World Health Organization technical report series. 2003;916:i-viii, 1-149, backcover.
4.   Lashen H, Fear K, Sturdee DW. Obesity is associated with increased risk of first trimester and recurrent miscarriage: matched case-control study. Hum Reprod. 2004 Jul;19(7):1644-6.
5.   Watkins ML, Rasmussen SA, Honein MA, Botto LD, Moore CA. Maternal obesity and risk for birth defects. Pediatrics. 2003 May;111(5 Pt 2):1152-8.
6.   McDonald SD, Han Z, Mulla S, Beyene J. Overweight and obesity in mothers and risk of preterm birth and low birth weight infants: systematic review and meta-analyses. BMJ. 2010;341:c3428.
7.   Chu SY, Kim SY, Lau J, Schmid CH, Dietz PM, Callaghan WM, et al. Maternal obesity and risk of stillbirth: a metaanalysis. Am J Obstet Gynecol. 2007 Sep;197(3):223-8.
8.   Kristensen J, Vestergaard M, Wisborg K, Kesmodel U, Secher NJ. Pre-pregnancy weight and the risk of stillbirth and neonatal death. BJOG. 2005 Apr;112(4):403-8.
9.   O'Brien TE, Ray JG, Chan WS. Maternal body mass index and the risk of preeclampsia: a systematic overview. Epidemiology. 2003 May;14(3):368-74.
10.   Jacobsen AF, Skjeldestad FE, Sandset PM. Ante- and postnatal risk factors of venous thrombosis: a hospital-based case-control study. Journal of thrombosis and haemostasis : JTH. 2008 Jun;6(6):905-12.
11.   Sebire NJ, Jolly M, Harris JP, Wadsworth J, Joffe M, Beard RW, et al. Maternal obesity and pregnancy outcome: a study of 287,213 pregnancies in London. Int J Obes Relat Metab Disord. 2001 Aug;25(8):1175-82.
12.   Denison F, Norrie G, Graham B, Lynch J, Harper N, Reynolds R. Increased maternal BMI is associated with an increased risk of minor complications during pregnancy with consequent cost implications. BJOG. 2009 Oct;116(11):1467-72. Epub 2009 Jun 4.
13.   Denison F, Price J, Graham C, Wild S, Liston W. Maternal obesity, length of gestation, risk of postdates pregnancy and spontaneous onset of labour at term. BJOG: An International Journal of Obstetrics & Gynaecology. 2008;115:720-5.
14.   Knight M TD, Kenyon S, Shakespeare J, Gray R, Kurinczuk JJ (Eds.) on behalf of MBRRACE-UK. Saving Lives, Improving Mothers' Care - Surveillance of maternal deaths in the UK 2011-13 and lessons learned to inform maternity care from the UK and Ireland Confidential Enquiries into Maternal Deaths and Morbidity 2009-13. National Perinatal Epidemiology Unit, University of Oxford. 2015.
15.   Curhan GC, Chertow GM, Willett WC, Spiegelman D, Colditz GA, Manson JE, et al. Birth weight and adult hypertension and obesity in women. Circulation. 1996 Sep 15;94(6):1310-5.
16.   Curhan GC, Willett WC, Rimm EB, Spiegelman D, Ascherio AL, Stampfer MJ. Birth weight and adult hypertension, diabetes mellitus, and obesity in US men. Circulation. 1996 Dec 15;94(12):3246-50.

17. Parsons TJ, Power C, Logan S, Summerbell CD. Childhood predictors of adult obesity: a systematic review. Int J Obes Relat Metab Disord. 1999 Nov;23 Suppl 8:S1-107.

18. Reynolds RM, Allan KM, Raja EA, Bhattacharya S, McNeill G, Hannaford PC, et al. Maternal obesity during pregnancy and premature mortality from cardiovascular event in adult offspring: follow-up of 1 323 275 person years. BMJ. 2013;347:f4539.

19. Pedersen J. Weight and length at birth of infants of diabetic mothers. Acta endocrinologica. 1954 Aug;16(4):330-42.

20. Whitaker RC, Dietz WH. Role of the prenatal environment in the development of obesity. J Pediatr. 1998 May;132(5):768-76.

21. Armitage JA, Poston L, Taylor PD. Developmental origins of obesity and the metabolic syndrome: the role of maternal obesity. Front Horm Res. 2008;36:73-84.

22. Minge CE, Bennett BD, Norman RJ, Robker RL. Peroxisome proliferator-activated receptor-gamma agonist rosiglitazone reverses the adverse effects of diet-induced obesity on oocyte quality. Endocrinology. 2008 May;149(5):2646-56.

23. Khan IY, Dekou V, Douglas G, Jensen R, Hanson MA, Poston L, et al. A high-fat diet during rat pregnancy or suckling induces cardiovascular dysfunction in adult offspring. Am J Physiol Regul Integr Comp Physiol. 2005 Jan;288(1):R127-33.

24. Bayol SA, Simbi BH, Bertrand JA, Stickland NC. Offspring from mothers fed a 'junk food' diet in pregnancy and lactation exhibit exacerbated adiposity that is more pronounced in females. The Journal of physiology. 2008 Jul 01;586(13):3219-30.

25. Howie GJ, Sloboda DM, Kamal T, Vickers MH. Maternal nutritional history predicts obesity in adult offspring independent of postnatal diet. The Journal of physiology. 2009 Feb 15;587(Pt 4):905-15.

26. Smith NA, McAuliffe FM, Quinn K, Lonergan P, Evans AC. Transient high glycaemic intake in the last trimester of pregnancy increases offspring birthweight and postnatal growth rate in sheep: a randomised control trial. Bjog. 2009 Jun;116(7):975-83.

27. Bayol SA, Macharia R, Farrington SJ, Simbi BH, Stickland NC. Evidence that a maternal "junk food" diet during pregnancy and lactation can reduce muscle force in offspring. Eur J Nutr. 2009 Feb;48(1):62-5.

28. Bayol SA, Simbi BH, Stickland NC. A maternal cafeteria diet during gestation and lactation promotes adiposity and impairs skeletal muscle development and metabolism in rat offspring at weaning. The Journal of physiology. 2005 Sep 15;567(Pt 3):951-61.

29. Tong JF, Yan X, Zhu MJ, Ford SP, Nathanielsz PW, Du M. Maternal obesity downregulates myogenesis and beta-catenin signaling in fetal skeletal muscle. Am J Physiol Endocrinol Metab. 2009 Apr;296(4):E917-24.

30. Yan X, Zhu MJ, Xu W, Tong JF, Ford SP, Nathanielsz PW, et al. Up-regulation of Toll-like receptor 4/nuclear factor-kappaB signaling is associated with enhanced adipogenesis and insulin resistance in fetal skeletal muscle of obese sheep at late gestation. Endocrinology. 2010 Jan;151(1):380-7.

31. Zhu MJ, Han B, Tong J, Ma C, Kimzey JM, Underwood KR, et al. AMP-activated protein kinase signalling pathways are down regulated and skeletal muscle development impaired in fetuses of obese, over-nourished sheep. The Journal of physiology. 2008 May 15;586(10):2651-64.

32.   Shelley P, Martin-Gronert MS, Rowlerson A, Poston L, Heales SJ, Hargreaves IP, et al. Altered skeletal muscle insulin signaling and mitochondrial complex II-III linked activity in adult offspring of obese mice. Am J Physiol Regul Integr Comp Physiol. 2009 Sep;297(3):R675-81.

33.   Samuelsson AM, Matthews PA, Argenton M, Christie MR, McConnell JM, Jansen EH, et al. Diet-induced obesity in female mice leads to offspring hyperphagia, adiposity, hypertension, and insulin resistance: a novel murine model of developmental programming. Hypertension. 2008 Feb;51(2):383-92.

34.   Bayol SA, Farrington SJ, Stickland NC. A maternal 'junk food' diet in pregnancy and lactation promotes an exacerbated taste for 'junk food' and a greater propensity for obesity in rat offspring. Br J Nutr. 2007 Oct;98(4):843-51.

35.   Muhlhausler BS, Adam CL, Marrocco EM, Findlay PA, Roberts CT, McFarlane JR, et al. Impact of glucose infusion on the structural and functional characteristics of adipose tissue and on hypothalamic gene expression for appetite regulatory neuropeptides in the sheep fetus during late gestation. The Journal of physiology. 2005 May 15;565(Pt 1):185-95.

36.   Surkan PJ, Hsieh CC, Johansson AL, Dickman PW, Cnattingius S. Reasons for increasing trends in large for gestational age births. Obstet Gynecol. 2004 Oct;104(4):720-6.

37.   Ogden CL, Carroll MD, Curtin LR, McDowell MA, Tabak CJ, Flegal KM. Prevalence of overweight and obesity in the United States, 1999-2004. Jama. 2006 Apr 05;295(13):1549-55.

38.   Kim J, Peterson KE, Scanlon KS, Fitzmaurice GM, Must A, Oken E, et al. Trends in overweight from 1980 through 2001 among preschool-aged children enrolled in a health maintenance organization. Obesity (Silver Spring). 2006 Jul;14(7):1107-12.

39.   Jensen DM, Damm P, Sorensen B, Molsted-Pedersen L, Westergaard JG, Ovesen P, et al. Pregnancy outcome and prepregnancy body mass index in 2459 glucose-tolerant Danish women. Am J Obstet Gynecol. 2003 Jul;189(1):239-44.

40.   Laitinen J, Power C, Jarvelin MR. Family social class, maternal body mass index, childhood body mass index, and age at menarche as predictors of adult obesity. Am J Clin Nutr. 2001 Sep;74(3):287-94.

41.   Lawlor DA, Smith GD, O'Callaghan M, Alati R, Mamun AA, Williams GM, et al. Epidemiologic evidence for the fetal overnutrition hypothesis: findings from the mater-university study of pregnancy and its outcomes. Am J Epidemiol. 2007 Feb 15;165(4):418-24.

42.   Li C, Kaur H, Choi WS, Huang TT, Lee RE, Ahluwalia JS. Additive interactions of maternal prepregnancy BMI and breast-feeding on childhood overweight. Obes Res. 2005 Feb;13(2):362-71.

43.   Whitaker RC. Predicting preschooler obesity at birth: the role of maternal obesity in early pregnancy. Pediatrics. 2004 Jul;114(1):e29-36.

44.   Reynolds RM, Osmond C, Phillips DI, Godfrey KM. Maternal BMI, parity, and pregnancy weight gain: influences on offspring adiposity in young adulthood. J Clin Endocrinol Metab. 2010 Dec;95(12):5365-9.

45.   Smith J, Cianflone K, Biron S, Hould FS, Lebel S, Marceau S, et al. Effects of maternal surgical weight loss in mothers on intergenerational transmission of obesity. J Clin Endocrinol Metab. 2009 Nov;94(11):4275-83.

46.   Fraser A, Tilling K, Macdonald-Wallis C, Sattar N, Brion MJ, Benfield L, et al. Association of maternal weight gain in pregnancy with offspring obesity and metabolic and vascular traits in childhood. Circulation. 2010 Jun 15;121(23):2557-64.

47.   Mamun AA, O'Callaghan M, Callaway L, Williams G, Najman J, Lawlor DA. Associations of gestational weight gain with offspring body mass index and blood pressure at 21 years of age: evidence from a birth cohort study. Circulation. 2009 Apr 07;119(13):1720-7.

48.   Moreira P, Padez C, Mourao-Carvalhal I, Rosado V. Maternal weight gain during pregnancy and overweight in Portuguese children. Int J Obes (Lond). 2007 Apr;31(4):608-14.

49.   Oken E, Rifas-Shiman SL, Field AE, Frazier AL, Gillman MW. Maternal gestational weight gain and offspring weight in adolescence. Obstet Gynecol. 2008 Nov;112(5):999-1006.

50.   Oken E, Taveras EM, Kleinman KP, Rich-Edwards JW, Gillman MW. Gestational weight gain and child adiposity at age 3 years. Am J Obstet Gynecol. 2007 Apr;196(4):322.e1-8.

51.   Wrotniak BH, Shults J, Butts S, Stettler N. Gestational weight gain and risk of overweight in the offspring at age 7 y in a multicenter, multiethnic cohort study. Am J Clin Nutr. 2008 Jun;87(6):1818-24.

52.   Bird A. Perceptions of epigenetics. Nature. 2007 May 24;447(7143):396-8.

53.   Gluckman PD, Hanson MA, Buklijas T, Low FM, Beedle AS. Epigenetic mechanisms that underpin metabolic and cardiovascular diseases. Nat Rev Endocrinol. 2009 Jul;5(7):401-8.

54.   Freeman DJ. Effects of maternal obesity on fetal growth and body composition: implications for programming and future health. Semin Fetal Neonatal Med. 2010 Apr;15(2):113-8.

55.   Aagaard-Tillery KM, Grove K, Bishop J, Ke X, Fu Q, McKnight R, et al. Developmental origins of disease and determinants of chromatin structure: maternal diet modifies the primate fetal epigenome. Journal of molecular endocrinology. 2008 Aug;41(2):91-102.

56.   Bouchard L, Thibault S, Guay SP, Santure M, Monpetit A, St-Pierre J, et al. Leptin gene epigenetic adaptation to impaired glucose metabolism during pregnancy. Diabetes Care. 2010 Nov;33(11):2436-41.

57.   Godfrey KM, Sheppard A, Gluckman PD, Lillycrop KA, Burdge GC, McLean C, et al. Epigenetic gene promoter methylation at birth is associated with child's later adiposity. Diabetes. 2011 May;60(5):1528-34.

58.   Crowther CA, Hiller JE, Moss JR, McPhee AJ, Jeffries WS, Robinson JS. Effect of treatment of gestational diabetes mellitus on pregnancy outcomes. N Engl J Med. 2005 Jun 16;352(24):2477-86.

59.   Landon MB, Spong CY, Thom E, Carpenter MW, Ramin SM, Casey B, et al. A multicenter, randomized trial of treatment for mild gestational diabetes. N Engl J Med. 2009 Oct 01;361(14):1339-48.

60.   Metzger BE, Lowe LP, Dyer AR, Trimble ER, Chaovarindr U, Coustan DR, et al. Hyperglycemia and adverse pregnancy outcomes. N Engl J Med. 2008 May 8;358(19):1991-2002.

61.   Group HSCR. Hyperglycemia and Adverse Pregnancy Outcome (HAPO) Study: associations with neonatal anthropometrics. Diabetes. 2009 Feb;58(2):453-9.

62. Catalano PM, Ehrenberg HM. The short- and long-term implications of maternal obesity on the mother and her offspring. BJOG. 2006 Oct;113(10):1126-33.

63. Catalano PM, Thomas A, Huston-Presley L, Amini SB. Increased fetal adiposity: a very sensitive marker of abnormal in utero development. Am J Obstet Gynecol. 2003 Dec;189(6):1698-704.

64. Dodd JM, Grivell RM, Crowther CA, Robinson JS. Antenatal interventions for overweight or obese pregnant women: a systematic review of randomised trials. BJOG. 2010 Oct;117(11):1316-26.

65. Oteng-Ntim E, Varma R, Croker H, Poston L, Doyle P. Lifestyle interventions for overweight and obese pregnant women to improve pregnancy outcome: systematic review and meta-analysis. BMC Med. 2012;10:47.

66. Dodd JM, Turnbull D, McPhee AJ, Deussen AR, Grivell RM, Yelland LN, et al. Antenatal lifestyle advice for women who are overweight or obese: LIMIT randomised trial. BMJ. 2014;348:g1285.

67. Vinter CA, Jensen DM, Ovesen P, Beck-Nielsen H, Jorgensen JS. The LiP (Lifestyle in Pregnancy) study: a randomized controlled trial of lifestyle intervention in 360 obese pregnant women. Diabetes Care. 2011 Dec;34(12):2502-7.

68. Poston L, Bell R, Croker H, Flynn AC, Godfrey KM, Goff L, et al. Effect of a behavioural intervention in obese pregnant women (the UPBEAT study): a multicentre, randomised controlled trial. The lancet Diabetes & endocrinology. 2015 Oct;3(10):767-77.

69. Syngelaki A, Nicolaides Kh Fau - Balani J, Balani J Fau - Hyer S, Hyer S Fau - Akolekar R, Akolekar R Fau - Kotecha R, Kotecha R Fau - Pastides A, et al. Metformin versus Placebo in Obese Pregnant Women without Diabetes Mellitus. N Engl J Med. 2016 20160204 DCOM- 20160212;374(5):434-43.

70. Witters LA. The blooming of the French lilac. J Clin Invest. 2001 Oct;108(8):1105-7.

71. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, et al. Role of AMP-activated protein kinase in mechanism of metformin action. J Clin Invest. 2001 Oct;108(8):1167-74.

72. El-Mir MY, Nogueira V, Fontaine E, Averet N, Rigoulet M, Leverve X. Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. The Journal of biological chemistry. 2000 Jan 7;275(1):223-8.

73. Owen MR, Doran E, Halestrap AP. Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. The Biochemical journal. 2000 Jun 15;348 Pt 3:607-14.

74. Foretz M, Hebrard S, Leclerc J, Zarrinpashneh E, Soty M, Mithieux G, et al. Metformin inhibits hepatic gluconeogenesis in mice independently of the LKB1/AMPK pathway via a decrease in hepatic energy state. J Clin Invest. 2010 Jul;120(7):2355-69.

75. National Institute for Health and Care Excellence. *Diabetes in Pregnancy: management from preconception to the postnatal period*. NICE guideline NG3.

76. Balsells M, Garcia-Patterson A, Sola I, Roque M, Gich I, Corcoy R. Glibenclamide, metformin, and insulin for the treatment of gestational diabetes: a systematic review and meta-analysis. BMJ. 2015;350:h102.

77.   Zhao L, Sheng X, Zhou S, Yang T, Ma L, Zhou Y, et al. Metformin versus insulin for gestational diabetes mellitus: a meta-analysis. Br J Clin Pharmacol. 2015 Apr 29.

78.   Singh KP, Rahimpanah F, Barclay M. Metformin for the management of gestational diabetes mellitus - A literature review. Aust N Z J Obstet Gynaecol. 2015 Apr 22.

79.   Beyuo T, Obed SA, Adjepong-Yamoah KK, Bugyei KA, Oppong SA, Marfoh K. Metformin versus Insulin in the Management of Pre-Gestational Diabetes Mellitus in Pregnancy and Gestational Diabetes Mellitus at the Korle Bu Teaching Hospital: A Randomized Clinical Trial. PLoS One. 2015;10(5):e0125712.

80.   George A, Mathews JE, Sam D, Beck M, Benjamin SJ, Abraham A, et al. Comparison of neonatal outcomes in women with gestational diabetes with moderate hyperglycaemia on metformin or glibenclamide - A randomised controlled trial. Aust N Z J Obstet Gynaecol. 2015 Feb;55(1):47-52.

81.   George A, Mathews JE, Sam D, Beck M, Benjamin SJ, Abraham A, et al. Comparison of neonatal outcomes in women with gestational diabetes with moderate hyperglycaemia on metformin or glibenclamide--a randomised controlled trial. Aust N Z J Obstet Gynaecol. 2015 Feb;55(1):47-52.

82.   Vanky E, Salvesen KA, Heimstad R, Fougner KJ, Romundstad P, Carlsen SM. Metformin reduces pregnancy complications without affecting androgen levels in pregnant polycystic ovary syndrome women: results of a randomized study. Hum Reprod. 2004 Aug;19(8):1734-40.

83.   Vanky E, Stridsklev S, Heimstad R, Romundstad P, Skogoy K, Kleggetveit O, et al. Metformin versus placebo from first trimester to delivery in polycystic ovary syndrome: a randomized, controlled multicenter study. J Clin Endocrinol Metab. 2010 Dec;95(12):E448-55.

84.   Boney CM, Verma A, Tucker R, Vohr BR. Metabolic syndrome in childhood: association with birth weight, maternal obesity, and gestational diabetes mellitus. Pediatrics. 2005 Mar;115(3):e290-6.

85.   Moher D, Hopewell S, Schulz KF, Montori V, Gotzsche PC, Devereaux PJ, et al. CONSORT 2010 explanation and elaboration: updated guidelines for reporting parallel group randomised trials. Bmj. 2010 Mar 23;340:c869.

86.   Scottish Health Survey. www.scotland.gov.uk. 2012.

87.   Health and Social Care Information Centre. www.hscic.gov.uk. 2014.

88.   Centre for Disease Control and Prevention: Overweight and Obesity. www.cdc.gov/obesity/data. 2012.

89.   Kanagalingam MG, Forouhi NG, Greer IA, Sattar N. Changes in booking body mass index over a decade: retrospective analysis from a Glasgow Maternity Hospital. BJOG. 2005 Oct;112(10):1431-3.

90.   Eastman NJ, Jackson E. Weight relationships in pregnancy. I. The bearing of maternal weight gain and pre-pregnancy weight on birth weight in full term pregnancies. Obstet Gynecol Surv. 1968 Nov;23(11):1003-25.

91.   Sewell MF, Huston-Presley L, Super DM, Catalano P. Increased neonatal fat mass, not lean body mass, is associated with maternal obesity. Am J Obstet Gynecol. 2006 Oct;195(4):1100-3.

92.   Hull HR, Dinger MK, Knehans AW, Thompson DM, Fields DA. Impact of maternal body mass index on neonate birthweight and body composition. Am J Obstet Gynecol. 2008 Apr;198(4):416 e1-6.

93. Humphreys RC. An analysis of the maternal and foetal weight factors in normal pregnancy. J Obstet Gynaecol Br Emp. 1954 Dec;61(6):764-71.

94. Heslehurst N, Simpson H, Ells LJ, Rankin J, Wilkinson J, Lang R, et al. The impact of maternal BMI status on pregnancy outcomes with immediate short-term obstetric resource implications: a meta-analysis. Obes Rev. 2008 Nov;9(6):635-83.

95. Dodd JM, Crowther CA, Robinson JS. Dietary and lifestyle interventions to limit weight gain during pregnancy for obese or overweight women: a systematic review. Acta Obstet Gynecol Scand. 2008;87(7):702-6.

96. Walsh JM, McGowan CA, Mahony R, Foley ME, McAuliffe FM. Low glycaemic index diet in pregnancy to prevent macrosomia (ROLO study): randomised control trial. BMJ. 2012;345:e5605.

97. Thangaratinam S, Rogozinska E, Jolly K, Glinkowski S, Roseboom T, Tomlinson JW, et al. Effects of interventions in pregnancy on maternal weight and obstetric outcomes: meta-analysis of randomised evidence. BMJ. 2012;344:e2088.

98. Scarpello JH, Howlett HC. Metformin therapy and clinical uses. Diab Vasc Dis Res. 2008 Sep;5(3):157-67.

99. NICE Guideline CG63: Diabetes in pregnancy. 2008.

100. Rowan JA, Hague WM, Gao W, Battin MR, Moore MP. Metformin versus insulin for the treatment of gestational diabetes. N Engl J Med. 2008 May 8;358(19):2003-15.

101. Rowan JA, Rush EC, Obolonkin V, Battin M, Wouldes T, Hague WM. Metformin in gestational diabetes: the offspring follow-up (MiG TOFU): body composition at 2 years of age. Diabetes Care. 2011 Oct;34(10):2279-84.

102. Nawaz FH, Khalid R, Naru T, Rizvi J. Does continuous use of metformin throughout pregnancy improve pregnancy outcomes in women with polycystic ovarian syndrome? J Obstet Gynaecol Res. 2008 Oct;34(5):832-7.

103. DIRECTIVE 2001/20/EC OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL
of 4 April 2001on the approximation of the laws, regulations and administrative provisions of the Member States relating to the implementation of good clinical practice in the conduct of clinical trials on medicinal products for human use. Official Journal of the European Communities. 2001.

104. Stewart FM, Freeman DJ, Ramsay JE, Greer IA, Caslake M, Ferrell WR. Longitudinal assessment of maternal endothelial function and markers of inflammation and placental function throughout pregnancy in lean and obese mothers. J Clin Endocrinol Metab. 2007 Mar;92(3):969-75.

105. Denison FC, Norwood P, Bhattacharya S, Duffy A, Mahmood T, Morris C, et al. Association between maternal body mass index during pregnancy, short-term morbidity, and increased health service costs: a population-based study. BJOG. 2014 Jan;121(1):72-81.

106. Johansson S, Villamor E, Altman M, Bonamy AK, Granath F, Cnattingius S. Maternal overweight and obesity in early pregnancy and risk of infant mortality: a population based cohort study in Sweden. BMJ. 2014;349:g6572.

107. Starling AP, Brinton JT, Glueck DH, Shapiro AL, Harrod CS, Lynch AM, et al. Associations of maternal BMI and gestational weight gain with neonatal adiposity in the Healthy Start study. Am J Clin Nutr. 2015 Feb;101(2):302-9.

108. Graversen L, Sorensen TI, Gerds TA, Petersen L, Sovio U, Kaakinen M, et al. Prediction of adolescent and adult adiposity outcomes from early life anthropometrics. Obesity (Silver Spring). 2015 Jan;23(1):162-9.

109. Scholl TO, Sowers M, Chen X, Lenders C. Maternal glucose concentration influences fetal growth, gestation, and pregnancy complications. Am J Epidemiol. 2001 Sep 15;154(6):514-20.

110. Eames AJ GR, Deussen AR, Hague W, Dodd JM. Meformin for women who are obese during pregnancy for improving maternal and infant outcomes. Cochrane Database Syst Rev. 2013;6(CD010564).

111. Chiswick CA, Reynolds RM, Denison FC, Whyte SA, Drake AJ, Newby DE, et al. Efficacy of metformin in pregnant obese women: a randomised controlled trial. BMJ Open. 2015;5(1):e006854.

112. Metzger BE, Gabbe SG, Persson B, Buchanan TA, Catalano PA, Damm P, et al. International association of diabetes and pregnancy study groups recommendations on the diagnosis and classification of hyperglycemia in pregnancy. Diabetes Care. 2010 Mar;33(3):676-82.

113. Bonellie S, Chalmers J, Gray R, Greer I, Jarvis S, Williams C. Centile charts for birthweight for gestational age for Scottish singleton births. BMC Pregnancy Childbirth. 2008;8:5.

114. Zhang Q, Zhao C, Davies MJ, Radican L, Seck T. Compliance and persistence with concomitant statin and oral antihyperglycemic therapy. The American journal of managed care. 2011 Nov;17(11):746-52.

115. Poston L, Bell R, Croker H, Flynn AC, Godfrey KM, Goff L, et al. Effect of a behavioural intervention in obese pregnant women (the UPBEAT study): a multicentre, randomised controlled trial. 20150921(2213-8595 (Electronic)).

116. Catov JM, Bodnar LM, Ness RB, Barron SJ, Roberts JM. Inflammation and dyslipidemia related to risk of spontaneous preterm birth. Am J Epidemiol. 2007 Dec 1;166(11):1312-9.

117. Xu L, Lee M, Jeyabalan A, Roberts JM. The relationship of hypovitaminosis D and IL-6 in preeclampsia. Am J Obstet Gynecol. 2014 Feb;210(2):149 e1-7.

118. Goldberg RB, Temprosa MG, Mather KJ, Orchard TJ, Kitabchi AE, Watson KE. Lifestyle and metformin interventions have a durable effect to lower CRP and tPA levels in the diabetes prevention program except in those who develop diabetes. Diabetes Care. 2014 Aug;37(8):2253-60.

119. Xu X, Du C, Zheng Q, Peng L, Sun Y. Effect of metformin on serum interleukin-6 levels in polycystic ovary syndrome: a systematic review. BMC women's health. 2014;14:93.

120. J P. Diabetes and Pregnancy: blood sugar of newborn infants. Danish Science Press. 1952.

121. Catalano PM, Hauguel-De Mouzon S. Is it time to revisit the Pedersen hypothesis in the face of the obesity epidemic? Am J Obstet Gynecol. 2011 Jun;204(6):479-87.

122. Salomaki H, Heinaniemi M, Vahatalo LH, Ailanen L, Eerola K, Ruohonen ST, et al. Prenatal metformin exposure in a maternal high fat diet mouse model alters the transcriptome and modifies the metabolic responses of the offspring. PLoS One. 2014;9(12):e115778.

123. Mills JL, Jovanovic L, Knopp R, Aarons J, Conley M, Park E, et al. Physiological reduction in fasting plasma glucose concentration in the first trimester

of normal pregnancy: the diabetes in early pregnancy study. Metabolism. 1998 Sep;47(9):1140-4.

124.   Catalano PM, Huston L, Amini SB, Kalhan SC. Longitudinal changes in glucose metabolism during pregnancy in obese women with normal glucose tolerance and gestational diabetes mellitus. Am J Obstet Gynecol. 1999 Apr;180(4):903-16.

125.   Catalano PM, Tyzbir ED, Wolfe RR, Calles J, Roman NM, Amini SB, et al. Carbohydrate metabolism during pregnancy in control subjects and women with gestational diabetes. Am J Physiol. 1993 Jan;264(1 Pt 1):E60-7.

126.   Catalano PM, Tyzbir ED, Roman NM, Amini SB, Sims EA. Longitudinal changes in insulin release and insulin resistance in nonobese pregnant women. Am J Obstet Gynecol. 1991 Dec;165(6 Pt 1):1667-72.

127.   Catalano PM, Tyzbir ED, Wolfe RR, Roman NM, Amini SB, Sims EA. Longitudinal changes in basal hepatic glucose production and suppression during insulin infusion in normal pregnant women. Am J Obstet Gynecol. 1992 Oct;167(4 Pt 1):913-9.

128.   Assel B, Rossi K, Kalhan S. Glucose metabolism during fasting through human pregnancy: comparison of tracer method with respiratory calorimetry. Am J Physiol. 1993 Sep;265(3 Pt 1):E351-6.

129.   Kalhan S, Rossi K, Gruca L, Burkett E, O'Brien A. Glucose turnover and gluconeogenesis in human pregnancy. J Clin Invest. 1997 Oct 1;100(7):1775-81.

130.   Kalhan SC, D'Angelo LJ, Savin SM, Adam PA. Glucose production in pregnant women at term gestation. Sources of glucose for human fetus. J Clin Invest. 1979 Mar;63(3):388-94.

131.   Homko CJ, Sivan E, Reece EA, Boden G. Fuel metabolism during pregnancy. Semin Reprod Endocrinol. 1999;17(2):119-25.

132.   Endo S, Maeda K, Suto M, Kaji T, Morine M, Kinoshita T, et al. Differences in insulin sensitivity in pregnant women with overweight and gestational diabetes mellitus. Gynecol Endocrinol. 2006 Jun;22(6):343-9.

133.   Sivan E, Chen X, Homko CJ, Reece EA, Boden G. Longitudinal study of carbohydrate metabolism in healthy obese pregnant women. Diabetes Care. 1997 Sep;20(9):1470-5.

134.   Forbes S, Barr SM, Reynolds RM, Semple S, Gray C, Andrew R, et al. Convergence in insulin resistance between very severely obese and lean women at the end of pregnancy. Diabetologia. 2015 Nov;58(11):2615-26.

135.   Definition and diagnosis of diabetes mellitus and intermediate hyperglycemia :
report of a WHO/IDF consultation. 2006.

136.   Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia. 1985 Jul;28(7):412-9.

137.   Katz A, Nambi SS, Mather K, Baron AD, Follmann DA, Sullivan G, et al. Quantitative insulin sensitivity check index: a simple, accurate method for assessing insulin sensitivity in humans. J Clin Endocrinol Metab. 2000 Jul;85(7):2402-10.

138.   Matsuda M, DeFronzo RA. Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp. Diabetes Care. 1999 Sep;22(9):1462-70.

139. Upreti R, Hughes KA, Livingstone DE, Gray CD, Minns FC, Macfarlane DP, et al. 5alpha-Reductase type 1 modulates insulin sensitivity in men. J Clin Endocrinol Metab. 2014(99):E1397-406.

140. Chiswick C, Reynolds RM, Denison F, Drake AJ, Forbes S, Newby DE, et al. Effect of metformin on maternal and fetal outcomes in obese pregnant women (EMPOWaR): a randomised, double-blind, placebo-controlled trial. Lancet Diabetes Enocrinol. 2015 20150921;3(10):778-86.

141. Giannarelli R, Aragona M, Coppelli A, Del Prato S. Reducing insulin resistance with metformin: the evidence today. Diabetes Metab. 2003 Sep;29(4 Pt 2):6S28-35.

142. Goodarzi MO, Bryer-Ash M. Metformin revisited: re-evaluation of its properties and role in the pharmacopoeia of modern antidiabetic agents. Diabetes Obes Metab. 2005 Nov;7(6):654-65.

143. Inzucchi SE, Maggs DG, Spollett GR, Page SL, Rife FS, Walton V, et al. Efficacy and metabolic effects of metformin and troglitazone in type II diabetes mellitus. N Engl J Med. 1998 Mar 26;338(13):867-72.

144. Christensen MM, Hojlund K, Hother-Nielsen O, Stage TB, Damkier P, Beck-Nielsen H, et al. Endogenous glucose production increases in response to metformin treatment in the glycogen-depleted state in humans: a randomised trial. Diabetologica. 2015 20151001;58(11):2494-502.

145. Romualdi D, De Cicco S, Gagliano D, Busacca M, Campagna G, Lanzone A, et al. How metformin acts in PCOS pregnant women: insights into insulin secretion and peripheral action at each trimester of gestation. Diabetes Care. 2013 Jun;36(6):1477-82.

146. Sivan E, Homko CJ, Chen X, Reece EA, Boden G. Effect of insulin on fat metabolism during and after normal pregnancy. Diabetes. 1999 Apr;48(4):834-8.

147. Hay WW, Jr., Sparks JW, Wilkening RB, Battaglia FC, Meschia G. Partition of maternal glucose production between conceptus and maternal tissues in sheep. Am J Physiol. 1983 Oct;245(4):E347-50.

148. Sawatzke AB, Norris AW, Spyropoulos F, Walsh SA, Acevedo MR, Hu S, et al. PET/CT imaging reveals unrivaled placental avidity for glucose compared to other tissues. Placenta. 2015 Feb;36(2):115-20.

149. Norris AW, Wang C, Yao J, Walsh SA, Sawatzke AB, Hu S, et al. Effect of insulin and dexamethasone on fetal assimilation of maternal glucose. Endocrinology. 2011 Jan;152(1):255-62.

150. Leonce J, Brockton N, Robinson S, Venkatesan S, Bannister P, Raman V, et al. Glucose production in the human placenta. Placenta. 2006 Apr;27 Suppl A:S103-8.

151. Elliott BD, Langer O, Schuessling F. Human placental glucose uptake and transport are not altered by the oral antihyperglycemic agent metformin. Am J Obstet Gynecol. 1997 Mar;176(3):527-30.

152. Charles B, Norris R, Xiao X, Hague W. Population pharmacokinetics of metformin in late pregnancy. Ther Drug Monit. 2006 Feb;28(1):67-72.

153. Vanky E, Zahlsen K, Spigset O, Carlsen SM. Placental passage of metformin in women with polycystic ovary syndrome. Fertil Steril. 2005 May;83(5):1575-8.

154. Hollenbeck CB, Johnston P, Varasteh BB, Chen YD, Reaven GM. Effects of metformin on glucose, insulin and lipid metabolism in patients with mild

hypertriglyceridaemia and non-insulin dependent diabetes by glucose tolerance test criteria. Diabete & metabolisme. 1991 Sep-Oct;17(5):483-9.

155. Wiernsperger NF, Bailey CJ. The antihyperglycaemic effect of metformin: therapeutic and cellular mechanisms. Drugs. 1999;58 Suppl 1:31-9; discussion 75-82.

156. Wu MS, Johnston P, Sheu WH, Hollenbeck CB, Jeng CY, Goldfine ID, et al. Effect of metformin on carbohydrate and lipoprotein metabolism in NIDDM patients. Diabetes Care. 1990 Jan;13(1):1-8.

157. Zhang T, He J, Xu C, Zu L, Jiang H, Pu S, et al. Mechanisms of metformin inhibiting lipolytic response to isoproterenol in primary rat adipocytes. Journal of molecular endocrinology. 2009 Jan;42(1):57-66.

158. Ramsay JE, Ferrell WR, Crawford L, Wallace AM, Greer IA, Sattar N. Maternal obesity is associated with dysregulation of metabolic, vascular, and inflammatory pathways. J Clin Endocrinol Metab. 2002 Sep;87(9):4231-7.

159. Diderholm B, Stridsberg M, Norden-Lindeberg S, Gustafsson J. Decreased maternal lipolysis in intrauterine growth restriction in the third trimester. BJOG. 2006 Feb;113(2):159-64.

160. Canoy D, Boekholdt SM, Wareham N, Luben R, Welch A, Bingham S, et al. Body fat distribution and risk of coronary heart disease in men and women in the European Prospective Investigation Into Cancer and Nutrition in Norfolk cohort: a population-based prospective study. Circulation. 2007 Dec 18;116(25):2933-43.

161. Kaess BM, Pedley A, Massaro JM, Murabito J, Hoffmann U, Fox CS. The ratio of visceral to subcutaneous fat, a metric of body fat distribution, is a unique correlate of cardiometabolic risk. Diabetologia. 2012 Oct;55(10):2622-30.

162. Kissebah AH, Krakower GR. Regional adiposity and morbidity. Physiol Rev. 1994 Oct;74(4):761-811.

163. Lapidus L, Bengtsson C, Larsson B, Pennert K, Rybo E, Sjostrom L. Distribution of adipose tissue and risk of cardiovascular disease and death: a 12 year follow up of participants in the population study of women in Gothenburg, Sweden. Br Med J (Clin Res Ed). 1984 Nov 10;289(6454):1257-61.

164. Bozzetto L, Prinster A, Mancini M, Giacco R, De Natale C, Salvatore M, et al. Liver fat in obesity: role of type 2 diabetes mellitus and adipose tissue distribution. Eur J Clin Invest. 2011 Jan;41(1):39-44.

165. Goodpaster BH, Wolf D. Skeletal muscle lipid accumulation in obesity, insulin resistance, and type 2 diabetes. Pediatr Diabetes. 2004 Dec;5(4):219-26.

166. Bjorntorp P. "Portal" adipose tissue as a generator of risk factors for cardiovascular disease and diabetes. Arteriosclerosis. 1990 Jul-Aug;10(4):493-6.

167. Jakobsen MU, Berentzen T, Sorensen TI, Overvad K. Abdominal obesity and fatty liver. Epidemiol Rev. 2007;29:77-87.

168. Siega-Riz AM, Herring AH, Carrier K, Evenson KR, Dole N, Deierlein A. Sociodemographic, perinatal, behavioral, and psychosocial predictors of weight retention at 3 and 12 months postpartum. Obesity (Silver Spring). 2010 Oct;18(10):1996-2003.

169. Rasmussen KM, Abrams B, Bodnar LM, Butte NF, Catalano PM, Maria Siega-Riz A. Recommendations for weight gain during pregnancy in the context of the obesity epidemic. Obstet Gynecol. 2010 Nov;116(5):1191-5.

170. Sohlstrom A, Wahlund LO, Forsum E. Total body fat and its distribution during human reproduction as assessed by magnetic resonance imaging. Basic Life Sci. 1993;60:181-4.

171. Stevens-Simon C, Thureen P, Barrett J, Stamm E. Skinfold caliper and ultrasound assessments of change in the distribution of subcutaneous fat during adolescent pregnancy. Int J Obes Relat Metab Disord. 2001 Sep;25(9):1340-5.

172. Soltani H, Fraser RB. A longitudinal study of maternal anthropometric changes in normal weight, overweight and obese women during pregnancy and postpartum. Br J Nutr. 2000 Jul;84(1):95-101.

173. Symonds ME, Mostyn A, Pearce S, Budge H, Stephenson T. Endocrine and nutritional regulation of fetal adipose tissue development. J Endocrinol. 2003 Dec;179(3):293-9.

174. Modi N, Murgasova D, Ruager-Martin R, Thomas EL, Hyde MJ, Gale C, et al. The influence of maternal body mass index on infant adiposity and hepatic lipid content. Pediatr Res. 2011 Sep;70(3):287-91.

175. Landon MB, Mintz MC, Gabbe SG. Sonographic evaluation of fetal abdominal growth: predictor of the large-for-gestational-age infant in pregnancies complicated by diabetes mellitus. Am J Obstet Gynecol. 1989 Jan;160(1):115-21.

176. Wladimiroff JW, Bloemsma CA, Wallenburg HC. Ultrasonic diagnosis of the large-for-dates infant. Obstet Gynecol. 1978 Sep;52(3):285-8.

177. Evans MI, Mukherjee AB, Schulman JD. Animal models of intrauterine growth retardation. Obstet Gynecol Surv. 1983 Apr;38(4):183-92.

178. Vintzileos AM, Neckles S, Campbell WA, Andreoli JW, Jr., Kaplan BM, Nochimson DJ. Fetal liver ultrasound measurements during normal pregnancy. Obstet Gynecol. 1985 Oct;66(4):477-80.

179. Wolfe HM, Sokol RJ, Martier SM, Zador IE. Maternal obesity: a potential source of error in sonographic prenatal diagnosis. Obstet Gynecol. 1990 Sep;76(3 Pt 1):339-42.

180. Patenaude Y, Pugash D, Lim K, Morin L, Bly S, Butt K, et al. The use of magnetic resonance imaging in the obstetric patient. J Obstet Gynaecol Can. 2014 Apr;36(4):349-63.

181. Strizek B, Jani JC, Mucyo E, De Keyzer F, Pauwels I, Ziane S, et al. Safety of MR Imaging at 1.5 T in Fetuses: A Retrospective Case-Control Study of Birth Weights and the Effects of Acoustic Noise. Radiology. 2015 May;275(2):530-7.

182. Gowland PA, De Wilde J. Temperature increase in the fetus due to radio frequency exposure during magnetic resonance scanning. Physics in medicine and biology. 2008 Nov 7;53(21):L15-8.

183. Hand JW, Li Y, Hajnal JV. Numerical study of RF exposure and the resulting temperature rise in the foetus during a magnetic resonance procedure. Physics in medicine and biology. 2010 Feb 21;55(4):913-30.

184. Clements H, Duncan KR, Fielding K, Gowland PA, Johnson IR, Baker PN. Infants exposed to MRI in utero have a normal paediatric assessment at 9 months of age. The British journal of radiology. 2000 Feb;73(866):190-4.

185. De Wilde JP, Rivers AW, Price DL. A review of the current use of magnetic resonance imaging in pregnancy and safety implications for the fetus. Prog Biophys Mol Biol. 2005 Feb-Apr;87(2-3):335-53.

186.   Baker PN, Johnson IR, Harvey PR, Gowland PA, Mansfield P. A three-year follow-up of children imaged in utero with echo-planar magnetic resonance. Am J Obstet Gynecol. 1994 Jan;170(1 Pt 1):32-3.

187.   Kok RD, de Vries MM, Heerschap A, van den Berg PP. Absence of harmful effects of magnetic resonance exposure at 1.5 T in utero during the third trimester of pregnancy: a follow-up study. Magnetic resonance imaging. 2004 Jul;22(6):851-4.

188.   Reeves MJ, Brandreth M, Whitby EH, Hart AR, Paley MN, Griffiths PD, et al. Neonatal cochlear function: measurement after exposure to acoustic noise during in utero MR imaging. Radiology. 2010 Dec;257(3):802-9.

189.   Hussain HK, Chenevert TL, Londy FJ, Gulani V, Swanson SD, McKenna BJ, et al. Hepatic fat fraction: MR imaging for quantitative measurement and display--early experience. Radiology. 2005 Dec;237(3):1048-55.

190.   Irwan R, Edens MA, Sijens PE. Assessment of the variations in fat content in normal liver using a fast MR imaging method in comparison with results obtained by spectroscopic imaging. Eur Radiol. 2008 Apr;18(4):806-13.

191.   Kim H, Taksali SE, Dufour S, Befroy D, Goodman TR, Petersen KF, et al. Comparative MR study of hepatic fat quantification using single-voxel proton spectroscopy, two-point dixon and three-point IDEAL. Magn Reson Med. 2008 Mar;59(3):521-7.

192.   Barr SM CC, Denison F, Copper A, Gray C, Semple S, Morton NM, Walker BR, Norman JE. Fat distribution and ectopic lipid distribution in morbidly obese pregnant women in the third trimester: MRI analyses. Arch Dis Child Fetal Neonatal Ed. 2012;97(Supplement 1):A34-A5.

193.   Vega GL, Chandalia M, Szczepaniak LS, Grundy SM. Metabolic correlates of nonalcoholic fatty liver in women and men. Hepatology. 2007 Sep;46(3):716-22.

194.   Szczepaniak LS, Nurenberg P, Leonard D, Browning JD, Reingold JS, Grundy S, et al. Magnetic resonance spectroscopy to measure hepatic triglyceride content: prevalence of hepatic steatosis in the general population. Am J Physiol Endocrinol Metab. 2005 Feb;288(2):E462-8.

195.   Sprung VS, Jones H, Pugh CJ, Aziz NF, Daousi C, Kemp GJ, et al. Endothelial dysfunction in hyperandrogenic polycystic ovary syndrome is not explained by either obesity or ectopic fat deposition. Clin Sci (Lond). 2014 Jan 1;126(1):67-74.

196.   Cusi K, Consoli A, DeFronzo RA. Metabolic effects of metformin on glucose and lactate metabolism in noninsulin-dependent diabetes mellitus. J Clin Endocrinol Metab. 1996 Nov;81(11):4059-67.

197.   Stumvoll M, Nurjhan N, Perriello G, Dailey G, Gerich JE. Metabolic effects of metformin in non-insulin-dependent diabetes mellitus. N Engl J Med. 1995 Aug 31;333(9):550-4.

198.   Lingvay I, Raskin P, Szczepaniak LS. Effect of insulin-metformin combination on hepatic steatosis in patients with type 2 diabetes. Journal of diabetes and its complications. 2007 May-Jun;21(3):137-42.

199.   Tiikkainen M, Hakkinen AM, Korsheninnikova E, Nyman T, Makimattila S, Yki-Jarvinen H. Effects of rosiglitazone and metformin on liver fat content, hepatic insulin resistance, insulin clearance, and gene expression in adipose tissue in patients with type 2 diabetes. Diabetes. 2004 Aug;53(8):2169-76.

200.   Herrera E, Amusquivar E. Lipid metabolism in the fetus and the newborn. Diabetes Metab Res Rev. 2000 May-Jun;16(3):202-10.

201.   Kannel WB, Brand N, Skinner JJ, Jr., Dawber TR, McNamara PM. The relation of adiposity to blood pressure and development of hypertension. The Framingham study. Ann Intern Med. 1967 Jul;67(1):48-59.
202.   Fisher JJ, Frey I. Pregnancy and parturition in the obese patient. Obstet Gynecol. 1958 Jan;11(1):92-4.
203.   Cedergren MI. Maternal morbid obesity and the risk of adverse pregnancy outcome. Obstet Gynecol. 2004 Feb;103(2):219-24.
204.   Bhattacharya S, Campbell DM, Liston WA, Bhattacharya S. Effect of Body Mass Index on pregnancy outcomes in nulliparous women delivering singleton babies. BMC Public Health. [Research Support, Non-U.S. Gov't].7:168.
205.   Seely EW, Ecker J. Clinical practice. Chronic hypertension in pregnancy. N Engl J Med. 2011 Aug 4;365(5):439-46.
206.   Jensen DM, Ovesen P, Beck-Nielsen H, Molsted-Pedersen L, Sorensen B, Vinter C, et al. Gestational weight gain and pregnancy outcomes in 481 obese glucose-tolerant women. Diabetes Care. 2005 Sep;28(9):2118-22.
207.   Duckitt K, Harrington D. Risk factors for pre-eclampsia at antenatal booking: systematic review of controlled studies. BMJ. 2005 Mar 12;330(7491):565.
208.   Libby G, Murphy DJ, McEwan NF, Greene SA, Forsyth JS, Chien PW, et al. Pre-eclampsia and the later development of type 2 diabetes in mothers and their children: an intergenerational study from the Walker cohort. Diabetologia. 2007 Mar;50(3):523-30.
209.   Sattar N, Gaw A, Packard CJ, Greer IA. Potential pathogenic roles of aberrant lipoprotein and fatty acid metabolism in pre-eclampsia. Br J Obstet Gynaecol. 1996 Jul;103(7):614-20.
210.   Roberts JM, Bodnar LM, Patrick TE, Powers RW. The Role of Obesity in Preeclampsia. Pregnancy Hypertens. 2011 Jan 1;1(1):6-16.
211.   Vita JA, Keaney JF, Jr. Endothelial function: a barometer for cardiovascular risk? Circulation. 2002 Aug 6;106(6):640-2.
212.   Endemann DH, Schiffrin EL. Endothelial dysfunction. J Am Soc Nephrol. 2004 Aug;15(8):1983-92.
213.   Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature. 1980 Nov 27;288(5789):373-6.
214.   Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature. 1993 Apr 29;362(6423):801-9.
215.   Engler MM, Engler MB, Malloy MJ, Chiu EY, Schloetter MC, Paul SM, et al. Antioxidant vitamins C and E improve endothelial function in children with hyperlipidemia: Endothelial Assessment of Risk from Lipids in Youth (EARLY) Trial. Circulation. 2003 Sep 2;108(9):1059-63.
216.   Raitakari M, Ilvonen T, Ahotupa M, Lehtimaki T, Harmoinen A, Suominen P, et al. Weight reduction with very-low-caloric diet and endothelial function in overweight adults: role of plasma glucose. Arterioscler Thromb Vasc Biol. 2004 Jan;24(1):124-8.
217.   Poston L. Endothelial dysfunction in pre-eclampsia. Pharmacol Rep. 2006;58 Suppl:69-74.
218.   Di Fulvio P, Pandolfi A, Formoso G, Di Silvestre S, Di Tomo P, Giardinelli A, et al. Features of endothelial dysfunction in umbilical cord vessels of women with gestational diabetes. Nutr Metab Cardiovasc Dis. 2014 Dec;24(12):1337-45.

219.   Nagi DK, Yudkin JS. Effects of metformin on insulin resistance, risk factors for cardiovascular disease, and plasminogen activator inhibitor in NIDDM subjects. A study of two ethnic groups. Diabetes Care. 1993 Apr;16(4):621-9.

220.   Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). UK Prospective Diabetes Study (UKPDS) Group. Lancet. 1998 Sep 12;352(9131):854-65.

221.   Diamanti-Kandarakis E, Alexandraki K, Protogerou A, Piperi C, Papamichael C, Aessopos A, et al. Metformin administration improves endothelial function in women with polycystic ovary syndrome. Eur J Endocrinol. 2005 May;152(5):749-56.

222.   Pitocco D, Zaccardi F, Tarzia P, Milo M, Scavone G, Rizzo P, et al. Metformin improves endothelial function in type 1 diabetic subjects: a pilot, placebo-controlled randomized study. Diabetes Obes Metab. 2013 May;15(5):427-31.

223.   Vitale C, Mercuro G, Cornoldi A, Fini M, Volterrani M, Rosano GM. Metformin improves endothelial function in patients with metabolic syndrome. J Intern Med. 2005 Sep;258(3):250-6.

224.   Flammer AJ, Luscher TF. Three decades of endothelium research: from the detection of nitric oxide to the everyday implementation of endothelial function measurements in cardiovascular diseases. Swiss medical weekly. 2010;140:w13122.

225.   Ludmer PL, Selwyn AP, Shook TL, Wayne RR, Mudge GH, Alexander RW, et al. Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. N Engl J Med. 1986 Oct 23;315(17):1046-51.

226.   Linder L, Kiowski W, Buhler FR, Luscher TF. Indirect evidence for release of endothelium-derived relaxing factor in human forearm circulation in vivo. Blunted response in essential hypertension. Circulation. 1990 Jun;81(6):1762-7.

227.   Celermajer DS, Sorensen KE, Gooch VM, Spiegelhalter DJ, Miller OI, Sullivan ID, et al. Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. Lancet. 1992 Nov 7;340(8828):1111-5.

228.   Benjamin N, Calver A, Collier J, Robinson B, Vallance P, Webb D. Measuring forearm blood flow and interpreting the responses to drugs and mediators. Hypertension. 1995 May;25(5):918-23.

229.   Axtell AL, Gomari FA, Cooke JP. Assessing endothelial vasodilator function with the Endo-PAT 2000. Journal of visualized experiments : JoVE. 2010(44).

230.   Nohria A, Gerhard-Herman M, Creager MA, Hurley S, Mitra D, Ganz P. Role of nitric oxide in the regulation of digital pulse volume amplitude in humans. Journal of applied physiology (Bethesda, Md : 1985). 2006 Aug;101(2):545-8.

231.   Joannides R, Richard V, Haefeli WE, Benoist A, Linder L, Luscher TF, et al. Role of nitric oxide in the regulation of the mechanical properties of peripheral conduit arteries in humans. Hypertension. 1997 Dec;30(6):1465-70.

232.   Joannides R, Richard V, Haefeli WE, Linder L, Luscher TF, Thuillez C. Role of basal and stimulated release of nitric oxide in the regulation of radial artery caliber in humans. Hypertension. 1995 Aug;26(2):327-31.

233.   Corretti M, Anderson T, Benjamin E, Celermajer D, Charbonneau F, Creager J, et al. Guidlines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilatation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. Journal of American College of Cardiology. 2002;39(2):257-65.

234.   Dorup I, Skajaa K, Sorensen KE. Normal pregnancy is associated with enhanced endothelium-dependent flow-mediated vasodilation. Am J Physiol. 1999 Mar;276(3 Pt 2):H821-5.

235.   Savvidou MD, Kametas NA, Donald AE, Nicolaides KH. Non-invasive assessment of endothelial function in normal pregnancy. Ultrasound Obstet Gynecol. 2000 Jun;15(6):502-7.

236.   Nelson-Piercy C. Handbook of Obstetric Medicine. Third ed: Informa Healthcare; 2007.

237.   Hashimoto M, Akishita M, Eto M, Ishikawa M, Kozaki K, Toba K, et al. Modulation of endothelium-dependent flow-mediated dilatation of the brachial artery by sex and menstrual cycle. Circulation. 1995 Dec 15;92(12):3431-5.

238.   Lieberman EH, Gerhard MD, Uehata A, Walsh BW, Selwyn AP, Ganz P, et al. Estrogen improves endothelium-dependent, flow-mediated vasodilation in postmenopausal women. Ann Intern Med. 1994 Dec 15;121(12):936-41.

239.   Williams DJ, Vallance PJ, Neild GH, Spencer JA, Imms FJ. Nitric oxide-mediated vasodilation in human pregnancy. Am J Physiol. 1997 Feb;272(2 Pt 2):H748-52.

240.   Romualdi D, Costantini B, Selvaggi L, Giuliani M, Cristello F, Macri F, et al. Metformin improves endothelial function in normoinsulinemic PCOS patients: a new prospective. Hum Reprod. 2008 Sep;23(9):2127-33.

241.   Nieuwenhuis-Ruifrok AE, Kuchenbecker WK, Hoek A, Middleton P, Norman RJ. Insulin sensitizing drugs for weight loss in women of reproductive age who are overweight or obese: systematic review and meta-analysis. Hum Reprod Update. 2009 Jan-Feb;15(1):57-68.

242.   Williams LK, Padhukasahasram B, Ahmedani BK, Peterson EL, Wells KE, Gonzalez Burchard E, et al. Differing effects of metformin on glycemic control by race-ethnicity. J Clin Endocrinol Metab. 2014 Sep;99(9):3160-8.

243.   Barker DJ, Osmond C. Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. Lancet. 1986 May 10;1(8489):1077-81.

244.   Huxley R, Owen CG, Whincup PH, Cook DG, Rich-Edwards J, Smith GD, et al. Is birth weight a risk factor for ischemic heart disease in later life? Am J Clin Nutr. 2007 May;85(5):1244-50.

245.   Barker DJ, Godfrey KM, Fall C, Osmond C, Winter PD, Shaheen SO. Relation of birth weight and childhood respiratory infection to adult lung function and death from chronic obstructive airways disease. Bmj. 1991 Sep 21;303(6804):671-5.

246.   Forsen T, Eriksson JG, Tuomilehto J, Teramo K, Osmond C, Barker DJ. Mother's weight in pregnancy and coronary heart disease in a cohort of Finnish men: follow up study. Bmj. 1997 Oct 04;315(7112):837-40.

247.   Osmond C, Barker DJ, Winter PD, Fall CH, Simmonds SJ. Early growth and death from cardiovascular disease in women. Bmj. 1993 Dec 11;307(6918):1519-24.

248.   McMillen IC, Robinson JS. Developmental origins of the metabolic syndrome: prediction, plasticity, and programming. Physiol Rev. 2005 Apr;85(2):571-633.

249.   Gluckman PD, Hanson MA. Living with the past: evolution, development, and patterns of disease. Science (New York, NY). 2004 Sep 17;305(5691):1733-6.