METFORMIN IN
GESTATIONAL DIABETES MELLITUS

By

DR MAY OO KHIN

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CONTENTS

List of Figures

Chapter 1

Chapter 2

Chapter 6

List of Tables

Chapter 1

Chapter 2

Comparison 1: Metformin vs Insulin

Comparison 2: Metformin vs Glibenclamide

Comparison 3: Metformin vs Di*et al*one

Chapter 6

List of Abbreviations

1C	one-carbon
ACC	acetyl CoA carboxylase
ACS	Acetyl CoA synthetase
Ado-Hcy	s-adenosylhomocysteine
Ado-Met	s-adenosylmethionine
AMP	adenosine monophosphate
AMPK	adenosine monophosphate kinase
apo	apolipoprotein
ATP	adenosine triphosphate
AZT/5-AZT	5-azacytidine
B12	vitamin B12
BMI	Body Mass Index
BSA	Bovine Serum Albumin
BWt	Birth weight
cAMP	cyclic adenosine monophosphate
Cbl	Cobalamin
cDNA	cyclic Deoxyribonucleic Acid
CE	Cholesterol Esters
ChoRE	Carbohydrate Response Elements
ChREBP	Carbohydate Responsive Binding Protein
CI	Confidence Interval
CPT I	Carnitine Palmitoyl Transferase I
CRP	C-reactive Protein
CSR	Caesarean Section Rates
Ct	cycle threshold
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic acid
DPP IV	Dipeptidyl peptidase IV

dTMP	deoxythymidine monophosphate
dUMP	deoxyuridine monophosphate
EDTA	ethylene-diamine-tetraacetic acid
EPA	eicosapentanoic acid
ER	endoplasmic reticulum
FAS	Fatty acid Synthase
FBS	Foetal Bovine Serum
FFA	Free Fatty Acids
FP2ase	Fructose-1,6-bisphosphatase
FPG	Fasting Plasma Glucose
G6P	Glucose-6-phosphate
G6Pase	Glucose-6-phosphatase
GA	Gestational Age
GDM	Gestational Diabetes Mellitus
GEH	George Eliot Hospital
GI	Gastrointestinal
GK	Glucokinase
GLP-1	Glucagon like peptide-1
GLUT	Glucose Transporter
GPAT	Glycerophophate Acyltransferase
GROW	Gestational Related Optimal Weight
GSK	Glycogen Synthase Kinase
HAPO	Hyperglycaemia and Adverse Pregnancy Outcomes
HbA1c	Glycated Haemoglobin
HDL	High Density Lipoprotein
HepG2	Human Hepatocellular carcinoma cell line
HGP	Hepatic Glucose Output
HMG-CoA	3-Hydroxy-3-methylglutaryl-CoA
HMGCR	3-Hydroxy-3-methylglutaryl-CoA Reductase
HOMA	Homeostasis Model Assessment
HOMA-IR	HOMA-insulin resistance

hPL	human Placental Lactogen
I2	Statistical Heterogeneity
IADPSG	International Association of Diabetes and Pregnancy Study Groups
IDF	International Diabetes Federation
IDL	Intermediate Density Lipoprotein
IRS	Insulin Receptor Substrate
IU	International Unit
IUGR	Intrauterine Growth Retardation
KCAC	Krebs citric acid cycle
LA	Long acting
LBW	Low Birthweight
LDL	Low Density Lipoprotein
LDLR	LDL recepter
LGA	Large for gestational age
L-PK	Liver-Phospho Kinase
LPL	Lipoprotein Lipase
MATE	Multidrug and Toxic Compound Extrusion
MEM	Modified Eagle's Medium
MMA	Methylmalanoic acid
MMCoA	Methylmalonyl CoA
MMCoAM	Methylmalonyl CoA Mutase
MOD	Mode of Delivery
mRNA	massenger ribonucleic acid
MS	Methionine Synthase
MTHF	Methyl Tetra Hydro Folate
MTP	Mitochondrial Transfer Protein
NAFLD	Non Alcoholic Fatty Liver Disease
NEFA	Non Esterified Fatty acids
NICE	National Institute of Clinical Excellence
NICU	Neonatal Intensive Care Unit

NPH	Neutral Protamine Hagedron
NPV	Negative Predictive Value
NRCT	Non-randomized controlled trials
OCT	Organic Cation Transporter
OGCT	oral glucose challenge test
OGTT	oral glucose tolerance test
OHA	oral hypoglycaemic agents
OR	odds ratio
PBS	Phosphate Buffer Saline
PBS-T	PBS-Tween
PCOS	Polycystic ovary syndrome
PE	pre-eclampsia
PEPCK	phosphoenol pyruvate carboxykinase
PGC	PPAR gamma coactivator
PI	Ponderal Index
PIH	Pregnancy-induced hypertension
PKA	Protein Kinase Synthase
PMAT	Plasma Monoamine Transporter
PP	postprandial
PPAR-γ	peroxisome proliferated activator receptors
PPV	positive predictive value
PVDF	polyvinylidine difluoride
RA	Rapid Acting
RBC	red blood cells
RCT	randomized controlled trials
RNA	ribonucleic acid
ROB	risk of bias tools
ROC	Receiver Operator Curve
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
S1P	Site-1-protease
S2P	Site-2-protease

SA	Short acting
SAM-e	s-adenosylmethionine
SCAP	SREBP cleavage-activating protein
SCBU	Special Baby Unit
SCD	Steroyl CoA decarboxylase
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	SDS-polyacrolamide gel electrophoresis
SEM	Standard Error of mean
SGA	Small for gestational age
SGLT	Sodium-Glucose linked transporter
SIGN	Scottish Intercollegiate Guideline Networks
SRE	Sterol regulatory elements
SREBF/SREBP	Sterol regulartory element binding factor/protein
STK11/LKB1	Serine Threonine Kinase 11
TC	Total cholesterol
TCA	Tricarboxylic cycle
TG	Triglyceride
tHcy	total homocysteine
THF	Tetrahydrofolate
TNF-α	Tumour Necrotic Factor-α
VLDL	very low density lipoprotein
WHO	World Health Organization
WMD	Weighted Mean Difference

DEDICATION

This thesis is dedicated to my mother, my father, my brother and my little sister for their never ending love and encouragement throughout my lifetime.

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DECLARATION

I hereby declare that this thesis is my own work except where I have otherwise stated. Information derived from work of others has been acknowledged by means of references. None of the work has been submitted for a degree at any other university.

Summary

Gestational diabetes mellitus (GDM) can affect up to 1 in 5 of pregnancies and is associated with adverse pregnancy outcomes including pre-eclampsia, neonatal hypoglycaemia, large for gestational age, increased adiposity and birth trauma. Good glycaemic control is the key to reduce these outcomes. Diet and lifestyle modification followed by insulin as necessary is the conventional type of management. Metformin is increasingly used in pregancy but with limited evidence, its role in GDM has not been well-established.

A systematic review including both randomized and non-randomized controlled studies have been conducted to evaluate the contemporary evidence of metformin in GDM. It is suggested that metformin in GDM could be a useful alternative to insulin and is regarded as the best oral anti-hyperglycaemic agent in GDM management currently. However, almost half of metformin-treated GDM patients required supplementary insulin to achieve target glucose levels (metformin failure). Women with higher metabolic risk factors are likely to develop metformin failure.

A clinical cohort of metformin-treated GDM is used to develop the predictive model to identify GDM women who are at risk of metformin failure. It has been found that women identified by new IADPSG and NICE 2015 fasting criteria are highly likely to develop metformin failure. It has also been established a number of algorithm based on various baseline characters of GDM women which will help primary healthcare physicians choose the best medication for GDM management.

One of the possible side-effects of metformin includes lowering of serum vitamin B12 levels whereas serum vitamin B12 deficiency during pregnancy which is associated with increased insulin resistance. It is reported that in low vitamin B12 state,

offspring's insulin resistance is found to be higher among women with high folate low B12 state. Hence, in order to fully appreciate the role of vitamin B12 deficiency in metformin failure, it is first necessary to understand the effects of folate in low vitamin B12 condition on pregnancy outcomes in GDM. It has also been found that in normal vitamin B12 GDM women, serum folate levels are negatively associated with plasma glucose levels but not low B12 state. This underlines the fact that in order for folate to have its role, it is important to have normal vitamin B12 levels.

Despite increasing use of metformin, it is not yet routine to check vitamin B12 levels before it is given. It is important to understand whether vitamin B12 has a role in metformin action. Thus, the mechanism by which vitamin B12 deficiency might interfere with metformin action was studied. In vitamin B12 deficient hepatocytes, metformin stimulation of AMPK was reduced which was followed by reduced downstream signalling in lipid metabolism. This effects were reversed by vitamin B12 supplementation. Thus, it is concluded that vitamin B12 deficiency could interfere with metformin action and before metformin is given, every GDM woman should be checked for serum vitamin B12 levels and should be supplemented if deficient.

Overall, vitamin B12 could play a critical role in GDM management and it is important for every GDM woman to have normal vitamin B12 levels.

CHAPTER 1

INTRODUCTION

1.1 Gestational Diabetes Mellitus (GDM)

In the late 1950s, the term “Gestational Diabetes Mellitus” was first developed by O’ Sullivan and his colleagues as “glucose intolerance of any degree which is first recognized during pregnancy” by using 100 gram oral glucose tolerance test (OGTT) (Kitzmiller *et al*, 2010). Although the definition does not change over time, different diagnostic cut-off values have been adapted in many countries using their local or national data. It should also be noted that because of lack of specificity in definition, the population might be mixed with undiagnosed type 2 diabetes (Alberti *et al* 1998; Metzger *et al* 2007). Basically, GDM is partly caused by placental hormonal disturbance resulting in impaired insulin sensitivity and deranged pancreatic beta cell function (Yogev *et al* 2008).

1.1.1 Epidemiology

1.1.1.1 Risk factors

It is understood that women with certain characteristics are at risk of impaired glucose tolerance during pregnancy. These characters include advanced maternal age, higher BMI, previous history of GDM, previous history of macrosomic baby, family history of type 2 diabetes and being a high-risk ethnic descent (Zhang 2010; NICE 2015; Galtier 2010). Like in type 2 diabetes, obesity plays a key role, modification of which could benefit the most on the overall prevalence. It is also well-recognized that pre-pregnancy obesity is invariably and directly related to high risk of GDM(Chu *et al* 2007). The risk factors associated with high risk of GDM were described in table 1.1.

Table 1.1 Risk factors of GDM (Galtier 2010)

Modifiable risk factor	Non-modifiable risk factor
• Physical activity • Diet • Cigarette smoking • Obesity • Socioeconomic factors	• Age • Ethnicity • Previous history of GDM • Family history of Type 2 diabetes • Maternal birth weight • Polycystic ovary syndrome • Obstetric history (history of macrosomia, intrauterine foetal death, multiparity) • Gestational weight gain • Multiple pregnancies

1.1.1.2 GDM and Ethnicity

The relationship between ethnicity and severity of insulin resistance during pregnancy seems to be independent of age and pre-pregnancy body weight (Yue *et al* 1996). Data on prevalences of GDM among different ethnic groups mostly come from metropolitan US-based studies. These studies invariably reported that the non-Hispanic European are at the lowest risk in studies which have diversity in population conducted at any time period(Lawrence 2010; Hunsberger *et al* 2010; Ferrara 2007; Savitz *et al* 2008). Savitz *et al* also reported that women from South Central Asia (Bangladesh, Indian, Pakistan) are at the remarkably highest risk of GDM among their population studied (Savitz *et al* 2008). Likewise, one UK-based study revealed that Indian women are at the highest risk (4.4%) followed by Southeast Asian (3.5%) and Black (1.5%) whereas White constituted only 0.4% of GDM population (Dornhorst *et al* 1992). GDM risk of being Black or African American is still controversial that some studies are found to

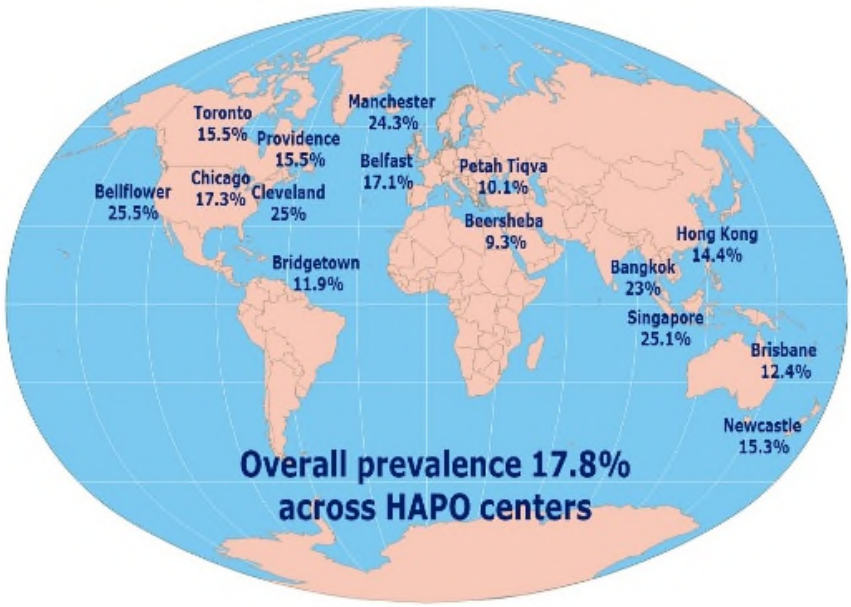
be the same (Ferrara 2007) while others are slightly higher than the non-Hispanic White (Savitz 2008; Dornhourst *et al* 1992). In addition, being a migrant or ancestry of migrants is another factor associated with high risk of GDM (Savitz *et al* 2008).

1.1.1.3 Prevalence of GDM

According to 2014 International Diabetes Federation (IDF) report, the prevalence varies from 10% to 25%, depending on ethnicity, diagnostic criteria, population being studied and diagnostic criteria being applied [15]. In UK where diabetes affect 2-5% of all pregnancies, almost fourth-fifths of adverse diabetes-related pregnancy outcomes could be attributable to gestational diabetes (IDF 2014). Despite global high prevalence and increasing trend of GDM worldwide, until now, internationally-agreed diagnostic glucose cut-off values for GDM have not yet been determined (Agarwal 2010; Simmons *et al* 2010; Cutchie *et al* 2006). Moreover, the methods of GDM screening also differ in that some countries use risk factor-based screening whereas others use 50 gram oral glucose challenge test (Agarwal 2010; Simmons *et al* 2010; Cutchie *et al* 2006). Generally the diagnostic values of impaired glucose level vary from 5.3 to 7 mmol/L of fasting plasma glucose (FPG) and 7.8 to 9.2 mmol/L of 2-hour postprandial (2hr PP) blood sugar using 75 (or) 100 grams OGTT [6, 16, 19-22] whereas in some countries, the contemporary criteria, recommended by World Health Organization (WHO) (Alberti *et al* 1998) & other international diabetes panels (Metzger 2007; IDF 2014) have been adopted. In 2010, International Association of Diabetes and Pregnancy Study Groups (IADPSG) have proposed the universal diagnostic criteria for GDM which have been developed from the pregnancy outcomes of Hyperglycemia and Adverse Pregnancy Outcome (HAPO) study (Hadar *et al* 2013).

According to IADPSG criteria, GDM is diagnosed if fasting glucose ≥ 5.1 mmol/L or 1 hour post prandial glucose ≥ 10.0 mmol/L or 2 hour post prandial glucose ≥8.5 mmol/L by using 75 gram OGTT test at 24-28 weeks gestation. If this criteria is adopted, the prevalence of GDM will be tripled (Gopalakrishnan *et al* 2015). The prevalences of GDM in 15 different countries by IADPSG criteria was described in Figure 1.1(Sacks *et al* 2012). Recently, the National Institute for Health and Care Excellence (NICE) has changed their GDM criteria which are fasting glucose ≥ 5.6 mmol/L or 2hour post prandial glucose ≥ 7.8 mmol/L by using 75 gram OGTT test(NICE NG-63 2015). The prevalence of GDM detected by this criteria is similar to that by IADPSG criteria (Meek *et al* 2015). Overall, it should be noted that there will be burgeoning number of GDM in very near future.

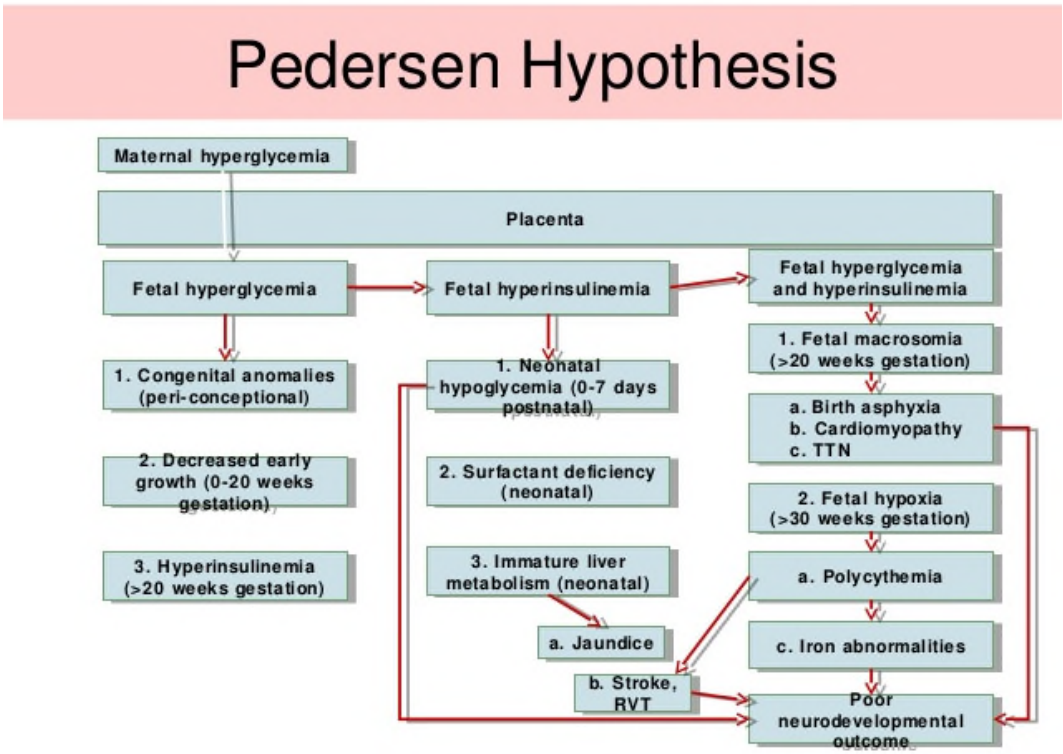
Figure 1.1 Prevalences of GDM by IADPSG criteria in 15 different countries (Data taken from Sacks DA *et al*. Diabetes care 2012; 35(3):526-528 and the figure taken from internet, shared by Khue NT in www.slideshare.net)



1.1.2 Consequences of GDM

It is understood that maternal hyperglycaemia, whether mild or severe, may impose a serious threat to both mothers and their babies. Moreover, a recent multicentre observational study has proved that there is continuous linear relationship between maternal hyperglycaemia and adverse pregnancy outcomes which is even below the diagnostic or target glucose levels used by different diabetes in pregnancy organizations (HAPO group 2008). Nevertheless, there is a great deal of evidence that the higher the maternal blood sugar level, the higher the risks of complication due to GDM (HAPO group 2008; Rowan *et al* 2010).

Figure 1.2 Pederson Hypothesis on consequences of hyperglycaemia during pregnancy (Figure taken from internet, shared by Khue NT in www.slideshare.net)



One of the major issues with GDM is the glycaemic impact on size of babies (HAPO group 2008). Macrosomia predisposes both mothers and newborns to other serious adverse perinatal outcomes. The term neonatal macrosomia usually refers to birth weight more than 4000g. Alternatively, it is defined as birth weight ≥ 90 percentiles for gestational age, also termed large for gestational age (LGA) (NICE NG-63 2015). By passing across the placenta, high blood sugar levels in mothers stimulate the fatal pancreatic beta cells to secrete more insulin. As insulin is an anabolic hormone, this will subsequently increase neonatal growth resulting in neonatal macrosomia and other associated risks (Pederson hypothesis) (Figure 1.2) (Singh *et al* 2008). This hyperglycaemic stimulation of foetal pancreatic insulin secretion can be compounded by gut-derived glucagon like peptide-1 (GLP-1), explained by Lois Jovanovic (Jovanovic 2007). These studies highlighted that when maternal hyperglycaemia was more than fatal renal threshold (<110 mg/dl), the overloaded glucose would be excreted into the amniotic fluid and >20 weeks gestation, the time when he could swallow, the glucose rich amniotic fluid would then enter the fatal gut and subsequently, would increase the gut-stimulated incretin secretion. He also mentioned that fatal β cell could be stimulated for hours despite being maternal hyperglycaemia for <1h/day (Jovanovic 2007). This vicious cycle together with transplacental glucose load results in an overfed, fat foetus. The foetal macrosomia together with subsequent increased incidence of shoulder dystocia and birth trauma is a major indication for instrumental delivery and caesarean section which impose a risk to mothers (Singh *et al* 2008; Wong *et al* 2011). At the same time, small for gestational age (SGA) is another important outcome related to pregnancy pertaining to GDM. It is usually defined as foetal birth weight less than 10 percentiles for gestational age (NICE NG-63 2015). SGA in GDM could be probably explained by placental vasculopathy

attributable to hyperglycaemia together with hyperlipidaemia (Dabelea 2010). Recently, it was found that maternal weight gain can have impact on both LGA and SGA in gestational diabetes (Barnes *et al* 2013).

Another major issue of GDM is neonatal hypoglycaemia which is attributable to the postnatal persistence of glucose-provoked foetal hyperinsulinaemia (Langer 2008). It is clinically defined as foetal glucose level < 2mmol/l or need for glucose injection (NICE NG-63 2015). Maternal glucose level at birth is the most influential factor of this outcome. The risk of neonatal hypoglycaemia among babies born to GDM is 10.38(95%CI 6.15 to 16.56) (Langer 2008). The lack of timely appropriate management of neonatal hypoglycaemia can lead to brain damage. However, data on risk of gestational diabetes on cognitive skills of offspring have not yet been well-documented (Veena *et al* 2010).

The other well-established risks of gestational diabetes are neonatal respiratory distress due to hyperinsulinaemia, neonatal jaundice probably explained by increased chance of birth trauma or increased red cell turnover because of foetal asphyxia, prematurity with underlying impending preeclampsia or foetal jeopardy, operative or assisted delivery (instrumentally assisted delivery or caesarean section), perinatal mortality, polyhydramnios, antepartum (infection related complications) and risk of macrosomia related postpartum complications, hypertensive complications of pregnancy and iatrogenic maternal hypoglycaemia (NICE NG-63 2015; Langer 2008).

Moreover, there are well-documented data of impact of maternal hyperglycaemia during pregnancy on offspring in long run. Firstly, it is suggested that untreated gestational diabetes could affect the intellectual abilities and mental development of the offspring (Langer 2007). This was found to have a relationship with raised level of

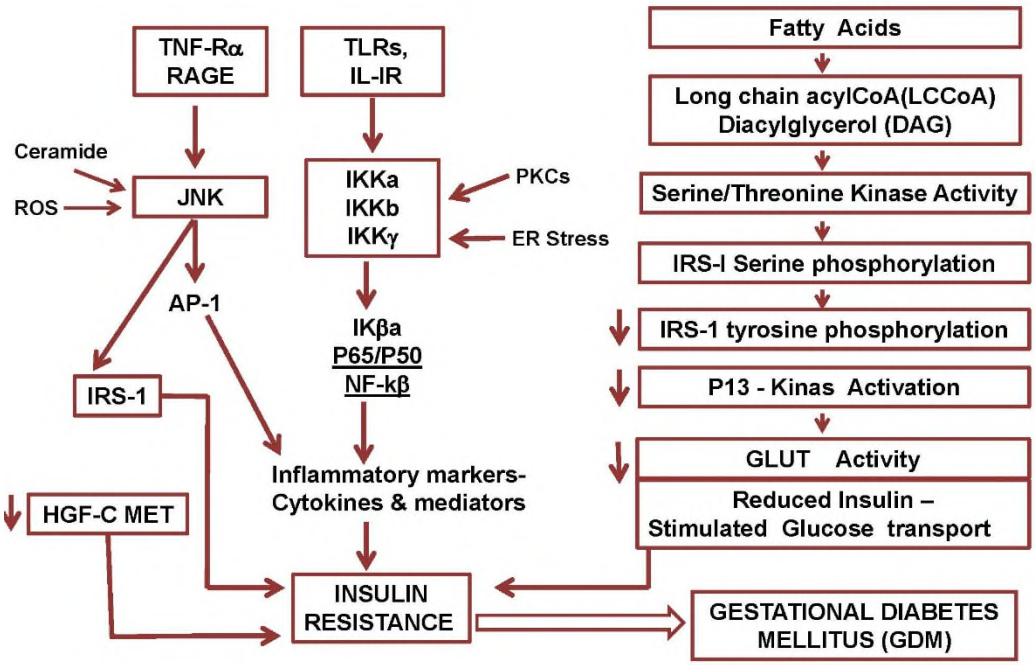
poor glycaemic measures (ketone bodies) in maternal serum in late pregnancy or history of spontaneous hypoglycaemia in neonatal period. Additionally, gestational diabetes associated adverse lipid profile could also impair the intelligence scales. Secondly, it is evident that babies born to GDM mothers have significant higher metabolic risk markers and higher chance of development of metabolic syndrome than those born to normal glucose tolerance, particularly those with large for gestational age (Boney 2005) and there is a clear link between exposure to increased insulin in intrauterine life to impaired insulin sensitivity in their adult life(Tam 2010).

Overall, GDM can have adverse effects on both immediate and long-term outcomes of pregnancy. The risks are imposed in GDM by hyperglycaemia and hyperinsulinaemia, its associated obesity and inflammatory cytokines. There is strong evidence that optimal and stable glycaemic control can reduce these risks(Landon 2009).

1.1.3 Pathogenesis of GDM

Gestational diabetes is universally accepted as a state of insulin insensitivity where there is impaired β-cell function to overcome abnormally high insulin resistance (Singh *et al* 2008). In fact, pregnancy itself is an insulin resistant state, resulting from diabetogenic placental factors. During pregnancy, impaired glucose tolerance can normally be compensated by enhanced beta-cell function. Hence, normally, it is not very common for pregnant mothers to develop GDM (Yogev *et al* 2008) Even though GDM has been widely believed as having moderate degree of similar pathophysiology as type 2 diabetes mellitus, in almost a-tenth of GDM, islet cell autoantibodies are found to be implicated (Retnakaran 2010). A brief description on inflammatory cytokines and signalling molecules implicated in GDM is described in Figure 1.3.

Figure 1.3 Inflammatory cytokines and insulin resistance in gestational diabetes (Data taken from Sholelson *et al* 2006, Qatanani *et al* 2007, Savage *et al* 2007; Figure taken from Omu AE. Gestational Diabetes - Causes, Diagnosis and Treatment 2014. Available at http://dx.doi.org/10.5772/56634)



Insulin resistance in GDM due to disturbance of placental factors could be at either insulin secretary or insulin performance stage: receptor level, post-receptor level or both (YOgev 2008). To our knowledge, the placental factors implicated in insulin resistance involve hormones – cortisol, human placental lactogen (hPL), human placental growth hormone and oestrogen & progesterone, inflammatory proteins – C-reactive protein (CRP), Tumour necrotic factor-alpha (TNF-α) and adipokines – adiponectin, leptin (Retnakaran 2010; McCurdy *et al* 2010; Omu 2014). Of these, high CRP and low adiponectin have been well-established as independent and significant risk factors of incident GDM (Retnakaran 2010) while placental hormones like cortisol and hPL are attributable to impaired pancreatic insulin secretion, inflammatory cytokines such as TNF-α interfere with post-receptor mechanisms, particularly at IRS level (Yogev *et al* 2008). As a result, the translocation of glucose transporter (GLUT 2) in liver or GLUT 4 in muscle and adipose tissues) to the cell surface is impaired with ultimate impact of hyperglycaemia (Yogev *et al* 1008; Retnakaran 2010; Omu 2014; Carrie *et al* 2010).

At the same time, any disturbance leading to accumulation of fatty acid in the cytosol can provoke insulin resistance, no matter whether increased free fatty acids may or may not be detected in the plasma (Ruderman *et al* 2006). During pregnancy, cholesterol and polyunsaturated fatty acids are essential for central nervous system development of foetus. Normal pregnancy adapts this high demand by increased fatty acid production through hormonal changes and increased placental transfer of essential fatty acid, highlighted by placental lipid transfer protein expression. Unfortunately, increased triglyceride production is invariably associated with GDM, even in the lean mothers, and even worse, GDM impairs vulnerability of peripheral tissues to oxidative stress imposed by raised fatty acids while increased triglycerides is related to high

incidence of preeclampsia (Knopp *et al* 2010). Taking all things into consideration, insulin resistance during pregnancy could be partly attributable to impact of high levels of free fatty acid on insulin sensitizing tissues.

1.1.4 Current management of GDM

The aim of management of gestational diabetes is to maintain blood glucose within safe limits for mother and fetus. The limitation is that there does not seem to be a clear threshold of glycemic level and risk of adverse pregnancy outcomes (HAPO group 2008). The risk seems to be linear without any clear cut-off point (HAPO group 2008). There is growing evidence that treating mild maternal hyperglycaemia (FPG <5.3 mmol/L) which is much lower than major international diagnostic blood glucose recommendation is associated with reduction in the incidence of adverse pregnancy outcomes (Landon *et al* 2009; Crowther *et al* 2006). On the other hand, it is stated that the risk of small-for-gestational-age infants is likely to be increased at much lower maternal mean capillary glucose level, specifically < 4.8 mmol/L (Metzger *et al* 2007). The currently agreed safe glucose levels vary between FPG of 3.8 to 6.1 mmol/L and postprandial level (either 1hr/2hr/3hr) of up to 9 mmol/L.

Though there might be conflict in diagnostic cut-off values, similar management guidelines have been applied to subsequent diagnosed GDM. They are managed first with medical nutritional therapy which is designed to meet nutritionally balanced diet for GDM mothers. If the diet therapy is failed to optimize glucose levels, the first universally agreed intervention is insulin, as insulin does not readily cross the placenta (Jovanovic *et al* 2007). It is undeniable that insulin is the effective pharmacological intervention for diabetes of any type and any degree to meet the glycemic control in pregnancy. However, there is concrete evidence that it has negative impact on

pregnancy outcomes as well as maternal satisfaction of using the drug (Alwan *et al* 2009; Rowan *et al* 2008). Use of insulin is associated with increase in incidence of maternal hypoglycaemia. It is also possible that the use of insulin in mothers can increase the risk of neonatal hypoglycaemia (Alwan *et al* 2009). Moreover, the high cost of insulin makes it impractical for implementation in resource poor healthcare system.

The application of IADPSG consensus criteria has been shown to benefit women at mild glycaemic level with reduction in adverse perinatal outcomes (Lapolla *et al* 2011). However, there is no randomized controlled trial (RCT) intervention evidence for treating women with GDM based on IADPSG criteria. It could be predicted that most countries will bring down their diagnostic criteria to lower glycaemic limit of GDM in near future. For these newly identified GDM who could presumably have lower baseline glucose levels than the current guidelines, insulin with hypoglycaemic risk would no longer be a good choice to address the challenge.

The risks and costs associated with use of insulin encourage searching for alternative available oral hypoglycaemic agents (OHA) in type 2 diabetes management. Up-to-date, glibenclamide (glyburide) and metformin have been appeared mostly as second-line (Alberti *et al* 1998; Metzger *et al* 2007; Cutchie *et al* 2006; CDACPGE committee 2003; ADA 2004; IDF 2009) and rarely as first-line therapy(NICE NG-63 2015; SIGN 2010). Glyburide is second-generation sulfonylurea drug which works by increasing insulin secretion and improving insulin sensitivity. It is understood to be as effective as insulin in the reduction of adverse neonatal outcomes in gestational diabetes mothers (Moretti *et al* 2008).

1.2 Metformin

Metformin is an ideal, first-line oral anti-hyperglycaemic agent widely used in Type 2 diabetes mellitus since 1957. Beyond its blood glucose lowering effects, it has other potential clinical roles in the prevention of progression to type 2 diabetes from pre-diabetes (impaired fasting glucose and impaired glucose tolerance)(Lilly *et al* 2009), improving fertility rates in women with polycystic ovary syndrome (PCOS), treating patients with liver steatosis and recent advances in prevention of carcinoma (Scarpello *et al* 2008).

1.2.1 Metformin in Pregnancy

Metformin is increasingly used during pregnancy recently in women with PCOS and those with GDM. However, the role of metformin during pregnancy is still not much significant owing to the concern that metformin does cross placenta and the drug level in the foetal compartment is as high as that in mother(Charles *et al* 2006). Even though the controversy continues, metformin has been frequently used in the pharmacological management of polycystic ovary syndrome. The increased conception rate in these relatively infertile women with metformin could underline the beneficial role of metformin in pregnancy. Although systematic review of randomized studies done by Lord *et al* have reported that metformin could reduce the risks of insulin resistance and have benefit on blood pressure and serum lipid levels in pregnancy (Lord *et al* 2003), one multicentre randomized trial did not find any association(Vanky *et al* 2010). At the same time, there are a few reports of metformin-associated increased perinatal risks. Hellmuth *et al* found greater numbers of stillbirth in metformin group whereas Coetzee and his group noticed high rate of neonatal jaundice in intrauterine metformin-exposed babies(Hellmuth *et al* 2000; Coetzee *et al* 1979). On the other

hand, Jean-Luc Ardilouze mentioned that congenital anomalies might not be an issue with the use of metformin in GDM saying that GDM is usually diagnosed in second trimester when embryogenesis and organogenesis of fetus have been completed (Ardilouze *et al* 2010). A meta-analysis of metformin in first trimester supported this hypothesis that metformin is not associated with major congenital anomalies with even better protection of some risks (Gilbert *et al* 2006). Recently, Tertti and his college reported that despite the comparable concentration of metformin in placental blood to maternal blood, there was no adverse pregnancy outcomes (Tertti *et al* 2014). Moreover, while NICE, SIGN, Canadian and Global Federation guidelines have included metformin as glucose lowering agents in GDM (NICE NG-63 2015; SIGN 2010; CDACPGE committee 2003; ADA 2004; IDF 2014), others yet to do so(Seshlah *et al* 2009). In Canadian guidelines, glibenclamide has been favoured over metformin, stating that metformin has higher failure rate, i.e., the proportion of GDM who have been achieved glycemic control with metformin alone therapy is less than that with glibenclamide alone therapy (CDACPGE committee 2003). Although metformin is included in a few guidelines of GDM management, the specific dosage recommendation has not yet been determined. In current GDM treatment, metformin is given initially as 500 mg or 250 mg together with meal once or twice daily, increasing the dose until optimal glycemic profiles have been achieved or maximum dose of 2500/2000mg/day, whichever comes first. This dosage guideline is taken from non-insulin dependent diabetes management (BNF 2015).

During the period of this PhD being undertaken, a number of meta-analyses in GDM has been published and it is found to be a beneficial drug for GDM (Balsells *et al* 2015; Kitwitee *et al* 2015; Su *et al* 2014; Gui *et al* 2013; Lautatzis *et al* 2013). In NICE 2015 GDM guidelines, metformin is also upgraded to give as first-line in GDM with

maternal consent (NICE NG-63 2015). So far, the evidences of metformin on pregnancy outcomes are still limited and as a promising drug with placental transfer, it is worthwhile to monitor metformin efficacy and safety on pregnancy outcomes, both in short and long run.

On the other hand, it has been reported that there is increased risk of prematurity (<37 weeks) with metformin therapy with the odds of 1.60 (95% confidence interval(CI):1.02-2.52) (Rowan *et al* 2008). The cause of this is not known. It could be possibly related to 1) gastrointestinal side-effects of metformin which mimic labour pain leading to delivery or 2) longer duration of suboptimal glycaemic control during escalation of metformin, thereby, promoting pre-eclampsia or similar adverse pregnancy outcomes necessitating immediate labour or 3) alteration of calcium metabolism in the gut, resulting in disturbance in uterine contractility (Bauman *et al* 2000). The finding reported by one small study, where metformin was ceased in metformin failure mothers and changed to insulin treatment rather than continuation with top-up insulin therapy until delivery, was that there was no difference in gestational age at delivery(Corbould *et al* 2013). This finding was controversial as this study included only 25 metformin users and did not report glycemic control after treatment. Nevertheless, if GDM were controlled more stringently and carefully after the diagnosis, the overall outcomes should be improved because all of poor outcomes in GDM are mostly due to poor glycemic control (HAPO group 2008).

Almost half of diet-failed GDM women who start with metformin therapy required supplementary insulin (referred as metformin failure in this thesis)(Rowan *et al* 2008; Ijas *et al* 2011, Goh 2t al 2011). Regarding metformin monotherapy in type 2 diabetes, 2-years evaluation of metformin done by Brown *et al* found that 42% of metformin-

treated type 2 diabetes needed additional glucose lowering alternatives within 5 years after successful glycemic control with metformin (Brown *et al* 2010). They also described that patients who develop diabetes at younger age, longer duration of diabetes or higher HbA1c could predict the higher risk of secondary metformin failure. It is stated that the failure rate could be as low as 12.2% if metformin is started within 3 months of diagnosis of diabetes. The data on the indicators of metformin failure in GDM are limited. Some studies have found that metformin-treated women with GDM who needed insulin have higher BMI and are possibly more insulin resistant (higher fasting & postprandial glucose and fructosamine at diagnosis) (Ijas *et al* 2011; Tertti *et al* 2013; Spaulonci *et al* 2013). One small study reported that GDM with fasting glucose at OGTT ≤5.2 mmol/l can achieve optimal glycaemic levels with metformin alone [71]. Moreover, it could be assumed that being European ethnicity have lower risk of metformin failure than Hispanic or South Asians (Rowan *et al* 2008; Goh *et al* 2011; Moore *et al* 2010). Given the slower onset of action, if metformin is initiated early in pregnancy, there may be lesser failure rate. Thus, if the treatment strategy of metformin is developed based on the maternal characters at the time of diagnosis, it can ensure consistent proper glycaemic control by addition of appropriate insulin to those with high chance of treatment failure. The significance of GDM women with high failure rate remains an area to be explored. It is also stated that the cost analysis of metformin in GDM has shown that metformin is not as costly as insulin, the additional cost associated with metformin failure is likely to be much higher (Lai *et al* 2008). So far, there are few studies that described the association of maternal characters and metformin response in GDM.

1.2.2 Recognized risks of metformin

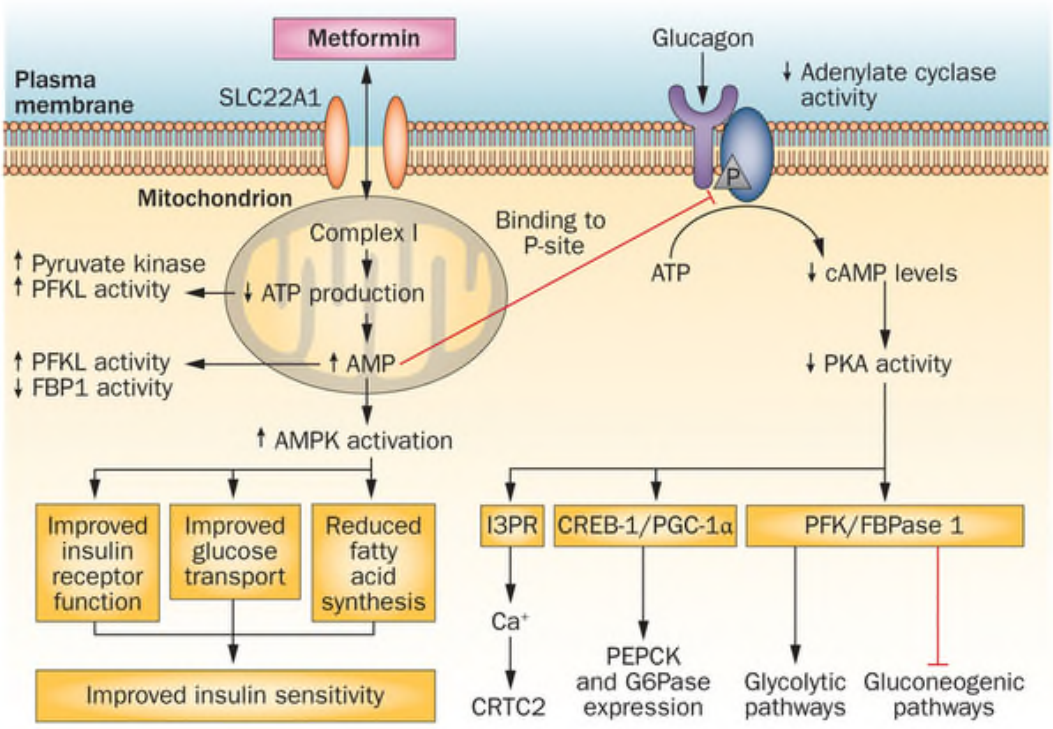
The adverse effects of metformin in type 2 diabetes are well-recorded. In current clinical practice, metformin is notorious for gastrointestinal side-effects - anorexia, nausea, vomiting, abdominal discomfort and diarrhoea, which usually occurs at the start of the treatment, but most patients can tolerate with dose reduction [80]. The discontinuation rate is also not common with only 5% of patients giving up treatment (Bailey *et al*. 2007a). It is stated that lactic acidosis, the most serious adverse effect of biguanide group, is rarely seen with metformin. For safety, metformin is not recommended to those with severe systemic hypoxia like uncompensated cardiac & respiratory failure, recent myocardial infarction, renal and hepatic dysfunction (Bailey *et al* 2007b).

1.2.3 Mechanism of action of metformin

The mechanisms of blood glucose lowering action of metformin differ by organ types. It is reported that metformin reduces hepatic glucose output, insulin sensitizing effects in muscle and adipose tissue and preservation of pancreatic beta cell function(Bailey *et al* 2007c). Recently, there is also evidence that metformin stimulates GLP-1 secretion in animal studies (Yasuda *et al* 2002) and inhibits DPP IV (dipeptidyl peptidase IV: a degradation enzyme for GLP-1) activity in type 2 diabetes patients(Lindsay *et al* 2005). Despite strong efficacy of blood glucose lowering effects, it has very few effects on blood glucose levels in normoglycaemic patients and thus it has very few hypoglycaemic risks. It has beneficial effects on blood lipid levels, i.e., reduction in triglyceride and Low Density Lipoprotein (LDL) cholesterol as well as improvement of High Density Lipoprotein (HDL) cholesterol(Schulters *et al* 2003; Campbell *et al* 2007). Furthermore, as a weight neutral anti-diabetic agent, it also

benefits on body weight control which could be observed as early as 6 weeks(Wulffele *et al* 2004). In addition, it is also effective in reducing cardiovascular risks reported by in UK prospective diabetes group study (UKPDS)(UKPDS group 1998).

Figure 1.4 Mechanism of metformin action (Figure taken from Pernicova *et al*. Nature Reviews Endocrinology 2014; 10,143-156)



1.2.3.1 Molecular mechanisms of action of metformin

The molecular mechanisms by which metformin lowers blood glucose level have not been well understood. Current understanding of metformin action at cellular model was illustrated in figure 1.4. The up-to-date data have shown that metformin primarily works by inhibiting at complex I of respiratory chain, thereby increasing energy demand (i.e. increasing the ratio of adenosine monophosphate (AMP) by adenosine

triphosphate(ATP))(Owen *et al* 2000; Pernicova *et al* 2014). The resultant increase in AMP: ATP ratio activates the energy sensor AMPK (adenosine monophosphate-activated protein kinase) enzyme which is a master energy regulator of the cell. Tumour suppressor serine/threonine kinase 11(STK11/LKB1) is suggested to be the upstream of AMPK by transmission of signal from mitochondrial inhibition(Pernicova *et al* 2014; Xie *et al* 2008). It helps balance the energy within the cell that metformin-induced energy depletion is counterbalanced by increasing energy breakdown pathways while reducing energy utilizing pathways, thereby, improving the overall metabolic function of the cell (Zhou *et al* 2001). In liver, AMPK activation favours fatty acid degradation, together with blockage of its synthesis and thus, helps improve insulin sensitivity and reduction of hepatic glucose output (Zhou *et al* 2001). AMPK activation is also important for its cardioprotective effects by promoting nitric oxide synthesis and decreasing the production reactive oxygen species in vascular endothelial cells (Scarpello *et al* 2008). At the same time, AMPK enhances peripheral glucose uptakes by potentiating insulin sensitizing effects on translocation of GLUT-4 receptor to cell surface in skeletal muscle and adipose tissue (Lee *et al* 2011) as well as it improves glucose metabolism in enterocytes by enhancing abundance of glucose transporters SGLT-1 and GLUT 2 (Sakar *et al* 2010). Alternatively, a decrease in AMPK activity can lead to impaired insulin sensitivity by allowing lipid deposition in skeletal muscle, adipose tissue and pancreatic β cells (Ruderman *et al* 2006). In addition to that, AMPK involves in the synthesis of mitochondria by promoting the regulation of transcriptional co activator PGC 1α (Ruderman *et al* 2006; Pernicova *et al* 2014). Hence, impaired AMPK function may reduce its metabolic efficacy by reducing the number of mitochondria. The pharmacokinetics of metformin also plays important roles in metformin action. Metformin is actively carried across the

membrane by its organic cation transporters (OCTs) which thereby determine the availability of metformin at the site of action (Rena *et al* 2013). There are various members of OCTs in the body; for active absorption across enterocyte membrane (plasma membrane monoamine transporter [PMAT] and OCT3) and into hepatocyte (OCT1) as well as for active secretion into bile (multidrug and toxic compound extrusion-1 [MATE1]) and into renal tubules (OCT2 and MATE2) (Rena *et al* 2013). The lack of OCT1 in animal model showed significant reduction in metformin efficacy and hence, it highlights the liver as primary site of metformin action (Rena *et al* 2013).

In Liver, metformin exerts its anti-hyperglycaemic effects by decreasing hepatic glucose output (HGP) at fasting (Zhou *et al* 2001). It is stated that this effect occurs through AMPK-dependent and AMPK-independent mechanisms (Renal *et al* 2013). Metformin-induced LKB1-AMPK axis inhibits gluconeogenesis by decreased transcription/expression of gluconeogenesis genes such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6P) (Zhou *et al* 2001). At the same time, among metformin given to LKB1 and AMPK knockout mice, HGP inhibition was found to be related to depletion of hepatic energy charge (ATP), and suppression of gluconeogenesis which occurs through transcription-independent mechanism(Rena *et al* 2013). The mechanism is suggested by Miller *et al* that metformin can diminish the effects of its counter-regulatory hormone, glucagon, on adenylate cyclase, thereby, depletion of cyclic AMP (cAMP) and concomitant activation of cAMP-dependent protein kinase (Protein kinase A/PKA) (Miller *et al* 2013).

Moreover, AMPK activation by metformin in liver also reduces hepatic lipid accumulation (Zang *et al* 2004). Once activated, AMPK phosphorylates and inhibits

the activity of Acetyl CoA Carboxylase (ACC) and thereby activating fatty acid oxidation resulting in reduction of intracellular lipid content (Zang *et al* 2006) . ACC is a bidirectional and rate-limiting enzyme in lipid biosynthesis and is also cross-link between lipid oxidation and synthesis (Nguyen *et al* 2008). During fed state, the non-phosphorylated active ACC form converts acetyl CoA to malonyl CoA, a precursor for elongation in fatty acid synthesis. There is evidence that AMPK influences fatty acid metabolism where low AMPK activity favours pathways towards assembly of fatty acyl CoA in the cytosol whereas phosphorylated AMPK favours lipid oxidation by decreasing malonyl CoA biosynthesis which limits mitochondrial fatty acid entry (Ruderman *et al* 2006; Zhou *et al* 2001). Once phosphorylated, it activates fatty acid oxidation by inhibiting ACC while increasing the activity of malonyl CoA decarboxylase which converts malonyl CoA to succinyl CoA, an essential substrate for Krebs citric acid cycle (KCAC). Zhou *et al* have found that metformin-induced AMPK activation also suppresses the mRNA expression of transcription factors important for triglyceride and cholesterol biosynthesis (known as sterol regulatory element binding factors/SREBFs)(Zhou *et al* 2001). Moreover, it also decreases the expression of the major enzymes in lipid synthesis such as ACC and Fatty acid synthase (FAS) in triglyceride biosynthesis and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) in cholesterol biosynthesis (Zhou *et al* 2001; Lee *et al* 2012). As a result, metformin reduces hepatic lipid accumulation by increasing fatty acid degradation, together with blockage of its synthesis (Zang *et al* 2004).

1.2.4 Metformin and reduced vitamin B12 levels

It has been extensively studied that long-term metformin use in type2 diabetes is associated with significant reduction in vitamin B_{12} level (Ting *et al* 2006; Liu *et al* 2006; Sorich *et al* 2008; Carpentier *et al* 1975; de Jager *et al* 2010) although there are some observational studies which have found no association (Radfar *et al* 2011; Hermann *et al* 2001). In 1971, Tomkin and his co-workers demonstrated that 30% of type 2 diabetes patients on metformin for more than 2 years were at risk of reduction in vitamin B12 levels, and stopping of metformin could return the B12 level to normal within 1 month (Tomkin *et al* 1971). The metformin-induced lowering of vitamin B12 levels could be due to alteration in calcium level in the gut, gut bacterial flora or abnormal fat absorption (Bauman *et al* 2000).

The prevalence of metformin induced vitamin B12 reduction varies from 14-30% (Hermann *et al* 2001). In fact, vitamin B12 deficiency (<150pmol/l) is significantly associated with duration (> 3years) (Ting *et al* 2006) and current dose of metformin use (Ting *et al* 2006; Sorich *et al* 2008). One randomized trial over 52 months with metformin performed by Jager *et al* reported that metformin-related vitamin B12 reduction is progressive over time (de Jager *et al* 2010). Ting *et al* have also reported that an increase in 1gram/day dose of metformin significantly rises the risk of vitamin B12 deficiency by more than 2 times and that of vitamin B12 insufficiency (150-220 pmol/l) by almost 4 times (Ting *et al* 2006). Sorich *et al* have reported "a significant negative correlation" between current metformin dose and vitamin B12 level ($r= -0.27$, $p=0.02$)(Sorich *et al* 2008). Furthermore, the randomized controlled trial of Jager *et al* have reported the number needed to be treated for both Vitamin B12 deficiency and

insufficiency being 13.8 per 4.3 years (95% CI 43.5 to 8.3) and 8.9 per 4.3 years (95% CI 21.7 to 5.6)respectively(de Jager *et al* 2010).

The use of metformin for less than 6 months has been found in two randomized studies. Sahin *et al* studied the effect of metformin on Vitamin B12 level in type 2 diabetes over 6 weeks period where 850 mg bd per day of metformin was offered to newly diagnosed type 2 diabetes patients (Sahin *et al* 2007). Although the study showed clinically significant change in Vitamin B12 level (mean difference -20.2 (-5.3 to -45.7) pmol/l, it failed to prove statistical significance (p=0.119)(Sahin *et al* 2007). Another randomized study of high dose metformin (mean dose 2163 mg/day) over 16 weeks done by Wulffele *et al* demonstrated significant reduction in Vitamin B12 level[mean change -14(-4.2 to -24 pmol/l) ($p<0.0001$)](Wulffele *et al* 2003). Although studies have almost invariably reported metformin-induced fall in vitamin B12 level, clinical reports of vitamin B12 deficient neurological and haematological disorders are rare.

In addition to dose and duration of metformin, there are also other risk factors related to metformin-induced Vitamin B12 deficiency. Ting *et al* reported that being vegetarian increases the odds of metformin-induced Vitamin B12 deficiency and insufficiency although the reported confidence interval is wide (OR 16.2(95% CI 1.69, 154)) (Ting *et al* 2006). Moreover, it is found that 10-yr increment in age is significantly associated with both B12 deficiency and insufficiency with the odds of 1.36 (96% 1.08-1.69) and 1.6 (1.24 to 2.04) respectively. The effect of H2 receptor blockers, proton pump inhibitors and antibiotics on the reduction of B12 level is still controversial(Ting *et al* 2006; Sorich *et al* 2008; Tomkin *et al* 1971). In fact, the prevalence of vitamin B12 deficiency related to reduced dietary intake in type 2

diabetes population who are not exposed to metformin is as high as almost 50% and half of these patients could be corrected with oral vitamin B12 over 3 months (Jawa *et al* 2010).

At the same time, the use of metformin in type 2 diabetes is also associated with rise in plasma total homocysteine (tHcy) level and fall in folate level although the evidence has not yet been confirmed (Sahin *et al* 2007; Wulffele *et al* 2003; Pongchaidecha *et al* 2004). Jager *et al* found that metformin related folate reduction is dependent of body mass index and smoking status(de Jager *et al* 2010). The change in tHcy in metformin-treated type 2 diabetes is both vitamin B12 and folate dependent (Wulffele *et al* 2003). Kilicdag and colleagues have also found that metformin-induced rise in tHcy level could be counteracted by B group vitamins (vitamin B1, B6 and B12) and folic acid (Kilicdag *et al* 2005). Recently, Rowan and her group have also reported that short-term use of metformin in GDM women is associated with reduction in serum vitamin B12 levels. In summary, metformin is understood to be progressive decrease in vitamin B12 whereas its effects on folate and homocysteine are still left to be justified.

1.3 Micronutrients in Pregnancy

1.3.1 Vitamin B12

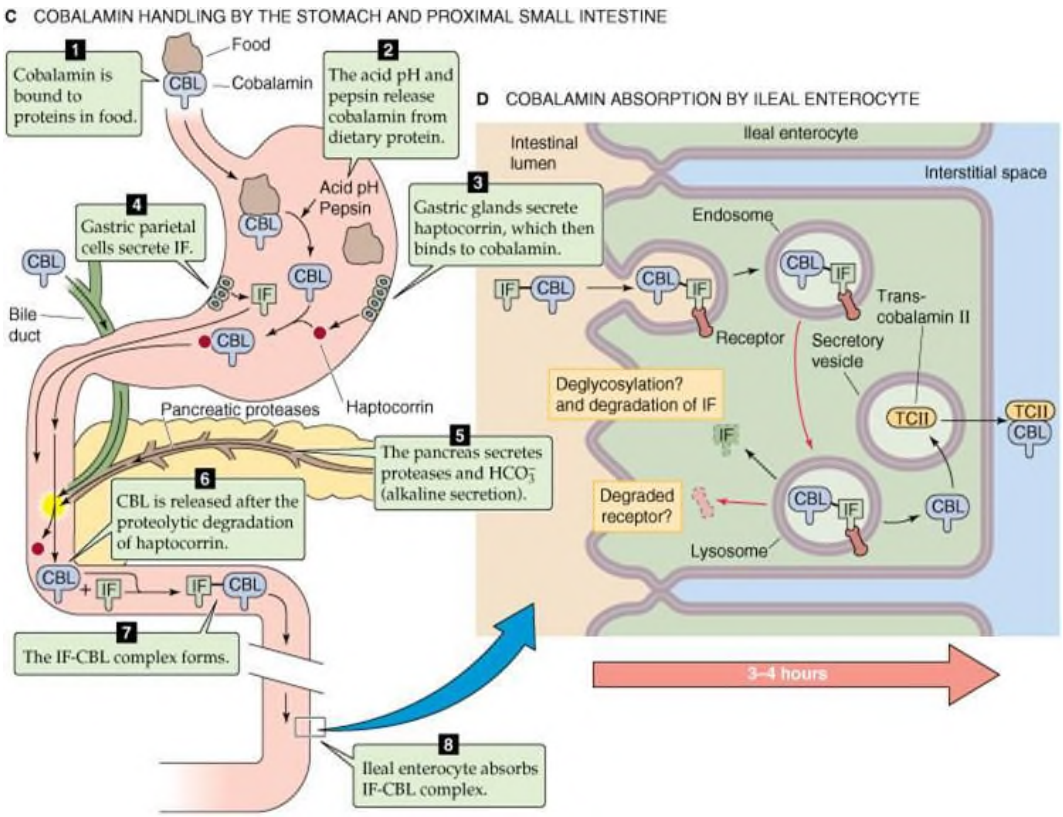
Vitamin B12 (Cobalamin) is water-soluble essential vitamin and it was recognized in the late 1940s (Depeint *et al* 2006). It contains cobalt ring and is synthesized only by bacteria or microorganisms. It presents in three forms in the diet as adenosylcobalamin (coenzyme B12), hydroxycobalamin and methylcobalamin. All three forms are found in meat and fish, especially liver, while dairy products have mostly hydroxycobalamin. Strict vegeterians are required to take it as a regular supplement. It is commercially available as cyanocobalamin which is then coverted to hydroxycobalamin and methylcobalamin. The daily requirement is very trivial (1-2ug/day) for a normal adult and increased to 2.6 ug/day during pregnancy. It can be stored in the liver adequately for 6 years and thus, deficiencies are rare.

1.3.1.1 Digestion, Absorption and Transport of Vitamin B12

An overview on vitamin B12 transport is illustrated in Figure 1.5. Firstly, cobalamin (Cbl) digestion begins in the stomach where pepsin helps releasing it from dietary protein in the acidic environment(Depeint *et al* 2006; Andres *et al* 2004). It then binds to salivary R binder which transports it to the small intestine. There, pancreatic enzymes digest the binder and it is then transferred to stomach-derived intrinsic factor (IF), forming IF/Cbl complex. With the help of specific IF receptor or cubilin, the complex then enters into the enterocyte by endocytosis. After that, it is dissociated in the lysosomes and the free Cbl is bound to newly synthesized transcobalamin II (TC II) which carries it to the portal system. In the liver, the TCII/Cbl complex enters by endocytosis by TCII specific receptor action and then it is freed again by proteolytic digestion in the lysosomes and endosomes. Cobalamin released to the cytosol is readily converted to methylcobalamin by methionine synthase reductase. The mechanism how vitamin B12 is transported to the mitochondria is still unknown. In the mitochondria, Cbl is reduced by reductase and is then transformed by adenosyl transferase and ATP to adenosylcobalamin which subsequently binds to methylmalonyl-CoA mutase (MMCoAM) as a coenzyme. Methylcobalamin, bound to transcobalamin I (TC I) or haptocorrin, are also released into the plasma and delivers to other peripheral tissues via asialoglycoprotein receptors. In the blood, 60-80% of Cbl are found in the form of methylcobalamin and 20% in adenosylcobalamin whereas 70% of Cbl in the liver represents adenosylcobalamin with 5% methylcobalamin(Bender 2003). Any physiological or pathological abnormalities in the function of gastrointestinal tract or plasma transport system can lead to diseases due to vitamin B12 deficiency. E.g. pernicious anaemia in the elderly due to impaired absorption and inborn error of

cobalamin metabolism due to defect in mitochondrial cobalamin binding protein(Ball 2004b).

Figure 1.5 Overview of vitamin B12 digestion, absorption and transport (Figure taken from www.b12g.org/book/export/html/1291)



1.3.2 Folic acid

In the late 1930s, folic acid was discovered as vitamin necessary to reduce anaemia during pregnancy. It is not synthesized in the body. Folates are available in the diet as 2 forms: dietary folates – from green leafy vegetables, eggs and legumes and fortified folates. Deficiency can result from poor nutritional intakes or malabsorption or high metabolic demands in conditions like pregnancy or anti-folate chemotherapy (e.g. methotrexate) (Ball 2004a).

Table 1.2. Daily requirement of folates in different age groups (adapted from Bailey *et al* 1999)

Group	Recommended Daily Allowance (µg/day)
Children and adolescents	
1-3	150
4-8	200
9-13	300
14-18	400
Adults	
≥19	400
Pregnant women	
All ages	600
Lactating women	
All ages	500

The daily requirement of folates in different age groups is shown in Table 1.2. These estimates are required to maintain normal red blood cell (RBC) folate levels. RBC folates are indicators of liver and other tissue storage of folates(Bailey *et al* 1999). Folates in the body function as a one-carbon (1C) carrier, required for methylation reactions which are important for deoxyribonucleic acid (DNA) synthesis and repair and gene expression. During pregnancy, adequate folate intake is vital for normal growth and development of fetus throughout gestation. Folate deficiency in pregnancy

can result in megaloblastic anaemia, neural tube defects and low birth weight infants (Depeint *et al* 2006).

1.3.2.1 Digestion, absorption and transport of folates

Folates in the diet are usually bound to polyglutamate proteins which need to be converted by proteases to monoglutamate forms before absorption in the proximal jejunum (Depeint *et al* 2006). Then, monoglutamates are transported to liver by H^+ co-transporter in the liver and folate receptor in other cells and mitochondria (Ball 2004a). Once in cytosol and mitochondria, they are elongated so that they can retain inside the cell.

1.3.3 Understanding cellular functions of Vitamin B12 and Folates

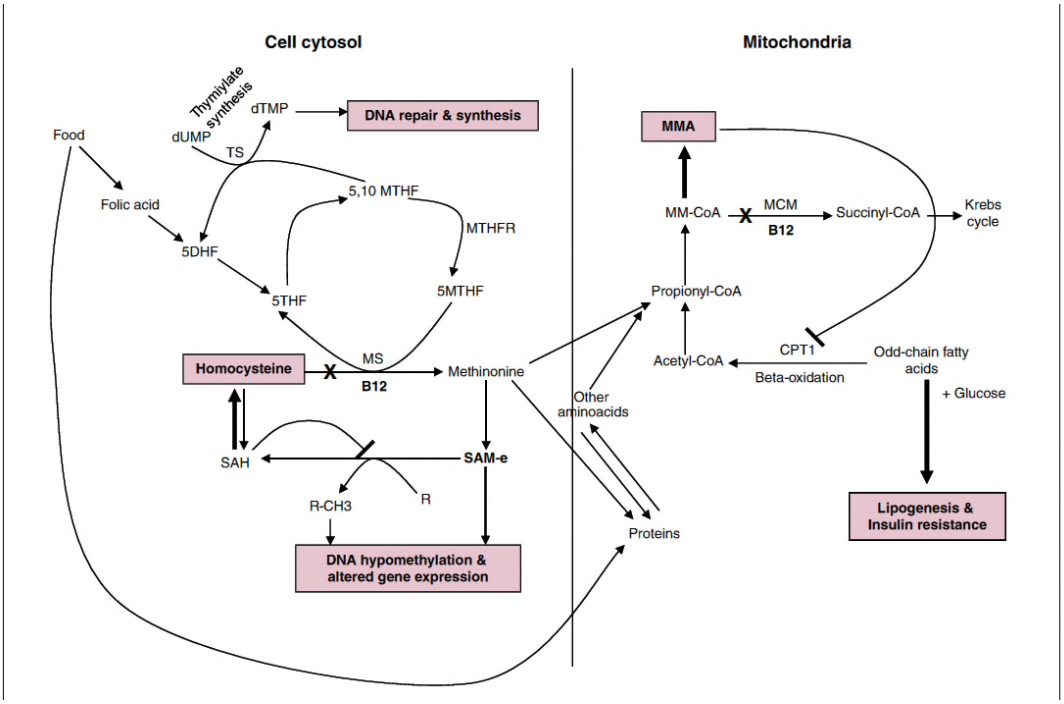
General overview of interaction of vitamin B12 and folates as a methylation reaction was given in Figure 1.5.

<u>In the cytosol</u>,

Vitamin B12 and folate play indispensible roles in one-carbon transfer pathway, which is important for DNA methylation and repair. Folate in the form of tetrahydrofolate (THF) plays as a carrier of 1C group from methy donars like serine inside the cell whereas vitamin B12 as methylcobalamine plays a cofactor in methyl group transfer. The reaction involves conversion of homocysteine to methionine by transfer of methyl group from methyltetrahydrofolate (MTHF) to the former, catalysed by methionine synthase (MS)(Kalhan 2009; Sardesai *et al* 2011). Methionine is subsequently converted to the active form called s-adenosylmethionine (SAM-e) which then passes over methyl group to methyl acceptor, required for "DNA methylation and gene expression" (Saravanan *et al* 2010). At the same time, demethylated MTHF/5-THF (5-

tetrahydrofolate) is remethylated back to form 5,10-methyltetrahydrofolate (5,10-MTHF) by the transfer of methyl group from deoxyuridine monophosphate (dUMP) and finally 5,10-MTHF is converted by 5,10-MTHFR to 5-MTHF, a readily available methyl donar for methionine formation. Meanwhile, the demethylated dUMP also is also transformed to deoxythymidine monophosphate (dTMP) which is important for nucleic acid formation and DNA repair (Sardesai *et al* 2011; Saravanan *et al* 2010; Nielsen *et al* 2012). Alternatively, either of functional vitamin deficiency can reduce methylation potential inside the cell and can impair one-carbon transfer, leading to hyperhomocysteinaemia, poor cardiovascular risk factor and "DNA hypomethylation and altered gene expression", contributing to "foetal programming"(Yajnik *et al* 2008). It has been reported that there is increased intracellular cholesterol accumulation in vitamin B12 deficiency due to hypomethylation and increased expressions of sterol regulatory element binding factor-1 (SREBF1), low density lipoprotein receptor (LDLR) and other cholesterol biosynthesis enzymes (Adaikalakoteswari *et al* 2015).

Figure 1.6 Model of vitamin B12 deficiency on cellular insulin resistance (taken from Saravanan P, Yajnik CS. 2010 in The British Journal of Diabetes and Vascular Disease)



dTMP = Thymidine monophosphate, dUMP = deoxyuridine monophosphate, TS = thymidylate synthase; MTHF = methyl tetrahydrofolate;MTHFR = methylene tetrahydrofolatereductase; DHF = dihydrofolate; THF = tetrahydrofolate; MS = methionine synthase; SAH = S-adenosylhomocysteine, SAM-e = S-adenosyl methionine; R = methyl acceptor; R- = methylated compound; MM-CoA = methylmalonyl-CoA mutase; CPT1 = cartinine palmitoyltransferase-1

In the mitochondria,

Vitamin B12 as adenosylcobalamin

Folates in the mitochondria are important for mitochondrial protein biosynthesis as well as glycine and serine cycling, both of which are important amino acid for purine and pyrimidine synthesis (Ball 2004b). At the same time, vitamin B12 plays as a coenzyme bound to MMCoAM for the synthesis of succinyl-CoA from methylmalonyl-CoA (MMCoA). MMCoA is converted from the propionyl-CoA which is a 3-carbon glucogenic fatty acid, end-product metabolites of methionine, valine, isoleucine or odd-chain fatty acids oxidation. The oxidation of long-chained fatty acids is initiated in the cytosol and then enters mitochondria as fatty-acyl CoA with the help of carnitine palmitoyltransferase I (CPT I), which is a rate-limiting step for fatty acid degradation and usually inhibited by high levels of malonyl-CoA. Once in mitochondria, they are then degraded to acetyl CoA and propionyl CoA. Propionyl CoA, is carboxylated to methylmalonylCoA which requires vitamin B12 to convert to succinyl-CoA (Sardesai *et al* 2011; Saravanan *et al* 2010; Nielsen *et al* 2012). Succinyl-CoA is an essential substrate for Tricarboxylic acid (TCA) cycle, an energy-generating cycle. In a state of B12 deficiency, MMCoA is pooled to form methylmaloic acid (MMA). MMA is structurally similar to malonyl CoA, which imitates malonyl CoA at CPT I level, thereby, interfering with fatty acyl Co A transfer and β-oxidation (Zierz *et al* 1987). Accumulation of fatty acyl CoA in the cytosol is diverted into extra mitochondrial lipid pathways producing lipid esters (e.g. diacylglycerol) and releasing metabolic stressors (e.g. reactive oxygen species) which provoke insulin resistance in liver and peripheral tissues (Ruderman *et al* 2006). However, the data of vitamin B12 deficiency on mitochondria is still limited. It is

stated that vitamin B12 is important for coenzyme Q biosynthesis, which is important for mitochondrial respiratory function (Ball 2004b). Therefore, it would be of worth presumption that mitochondria function might be impeded in vitamin B12 deficiency.

In addition to its involvement in insulin resistance, vitamin B12 is also important for optimal neuronal function in the sense that the aggregated MMCoA substitutes the role of malonyl CoA in fatty acid synthesis which is essential for generation of nervous tissue, resulting in impaired adaptability of neuronal function (Saravanan *et al* 2010). Moreover, the accumulated MMCoA, which is converting backwards to propionyl CoA (3-carbon CoA) may favour formation of odd-chain fatty acids, which also interferes with cell membrane integrity if being incorporate into membrane phospholipid synthesis (Sardesai 2011) By these means, B12 is indispensible for stabilization of myelin sheath membrane. There is also evidence that the aggregated MMCoA substitutes MMA in fatty acid in neuronal membrane and interferes with neuronal integrity and adaptability (Ball 2004b).

It has already mentioned that in B12 deficiency, homocysteine can be accumulated. High homocysteine level during pregnancy could probably be an explanation for insulin resistance in GDM (Guven *et al* 2006; Seghieri *et al* 2003). Homocysteine is "a thiol (SH)-containing amino acid", one of which pathways includes conversion to essential amino acid, methionine, by going through methylation reaction with the acceptance of methyl group from folate with the help of coenzyme vitamin B12(Hague *et al* 2003). As mentioned above, methionine acts as a methyl donor, transferring its methyl group to "methylated acceptors", that involves in DNA synthesis. High homocysteine level in pregnancy has been well-established as being responsible for neural tube defects, pre-eclampsia, and placental vasculopathy with its related early

pregnancy loss and abruptio placenta. Similar to its association with GDM, high homocysteine level is also found to be related to insulin resistance in polycystic ovary syndrome (Schachter *et al* 2003) and insulin resistance syndrome (Meigs *et al* 2001). Any disturbance in metabolism of homocysteine including B-group vitamins and folate deficiency as well as enzyme defect could result in high homocysteine level.

1.3.4 Transgenerational impact of vitamin B12 deficiency

The possible mechanisms how maternal B12 level relates to the long-term risk of B12-related metabolic diseases in offspring have been reviewed by Yajnik *et al* (Yajnik *et al* 2008) (Figure 1.6). They have explained that this may occur through either "foetal programming" or shared dietary pattern between mothers and offspring. If maternal vitamin B12 is not enough to supply methyl group for cellular differentiation of the embryo, it may interfere with protein synthesis, resulting in lower lean body mass. At the same time, disturbance in fatty acid synthesis may lead to unfavourable lipid distribution in offspring of B12 deficient mothers. Moreover, there is also evidence that newborn's B12 and homocysteine level defined by cord blood at birth is strongly predicted by maternal vitamin B12 level at labour (Guerra-Shinohara *et al* 2002). After birth, the newborns tend to share the same dietary pattern with their mothers and subsequently, they may have low B12 levels. Their low B12 levels will determine their body composition of adipose tissue. Therefore, like their moms, newborns of B12 deficient mothers are at risk of being fat.

In accordance with their explanations, Yajnik and his co-workers have also reported that newborns of B12 deficient high homocysteine mothers tend to be at risk of lower birth weight at birth (Yajnik et la 2005) and at 6 years more insulin resistant than their B12 sufficient comparators (Yajnik *et al* 2007). The increased maternal MMA level is

found to have significant association with central fat deposition and impaired glucose tolerance of offspring at 6 years of age (Yajnik *et al* 2007). The highest insulin resistance in the 6-year-olds was found in those with maternal lowest B12 but highest folate tertiles (Yajnik *et al* 2008). This group has also reported that low maternal B12 could have risks on offspring's' memory and attention span (Bhate *et al* 2008). It is evident that maternal B12 levels may influence not only foetal body composition but also neuronal function.

1.3.5 Impact of Low Vitamin B12 High Folates conditions

During pregnancy, there is high demand of B12 and folate for methylation reaction and DNA synthesis. Reduction in neural tube defects by folic acid supplementation has led to fortification of food in US since 1997. A decade later, it was found that high folate levels particularly in the state of vitamin B12 deficiency are associated with increased homocysteine and methylmalonic acid levels and increased incidence of anaemia and neurological impairment in the elderly (Selhub *et al* 2007; Ralph *et al* 2005; MacFarlane *et al* 2011). High folates are not normally recommended to pregnant women during pregnancy. However, in some cases like diabetic pregnancy which is highly associated with neural tube defects and in some countries where dietary folate deficiency are common, high folates of 5mg are given to pregnant women. So, in pregnancy folate deficiency is rarely seen. In fact, both folic acid and vitamin B12 are responsible for optimal development of neural tube (Kirke *et al* 1993). High homocysteine level could be implicated in B12 deficient mothers, even in adequate folate supply (Yajnik *et al* 2008).

As mentioned above, it is common to see pregnancy with low B12 and high folate states. During the past few years, there have been reports on impact of low B12 high

folate states on pregnancy outcomes. The interaction of folate level in B12 deficient pregnant women is found to have positive relationship with Homeostasis Model Assessment (HOMA) insulin resistance whereas the significant reverse relation is observed in non-deficient mothers (Krishnaveni *et al* 2009). Similarly, it has been reported that high folates low vitamin B12 states during pregnancy can increase the risk of infants with small-for-gestational-age (SGA) and low birth weight (LBW) (Dwarkanath *et al* 2013; Gadgil *et al* 2014). Moreover, this imbalance can also increase the incidence of gestational diabetes mellitus in mothers (Krishnaveni *et al* 2009). This might be due to the detrimental effect of B12 deficiency implicated high homocysteine level although the study failed to report homocysteine level. This association has been found to be obesity-related. However, this cross-sectional study was not able to establish the causal role of B12 deficiency in GDM. Recently, Krishnaveni and her group have found higher insulin resistance in the adolescent offspring born from mothers with higher folate concentrations, but not vitamin B12(Krishnaveni *et al* 2014). Thus, it should be noted that although folates are essential in pregnancy, high folate intakes can have detrimental effects on pregnancy outcomes, especially in the presence of vitamin B12 deficiency. The question of whether high folate or low vitamin B12 or combination can have these adverse outcomes are still controvertial.

1.3.6 Vitamin B12 deficiency and insulin resistance in clinical studies

It is suggested that the considerably higher diabetic risk of GDM in South Asians could be related to increased adipose tissue composition, compared to their European counterparts (Barnett *et al* 2006). A study on comparison of body fat composition in multi-ethnic UK children has showed that despite lower BMI in South Asians, their

proportion of adiposity, determined by skin fold thickness and waist circumference, is found to be significantly higher than the White (Nightingale *et al* 2011). This excess fat composition nature of South Asians could be explained by B12 insufficiency (Yajnik *et al* 2008). Owing to the lack of B12-abundant food such as meat and milk in their regular meal, the South Asians are found to have lower B12 level than the Europeans (Chambers *et al* 2000).

Similarly, a comparison study of insulin sensitivity among different ethnic groups has reported that insulin resistance is higher in South Asians and Asians in comparison to their Caucasian counterparts(Retnakaran *et al* 2006). In this study, it has also been found that being South Asian is modestly associated with pre-pregnancy body mass index (BMI) in contrast to strong relationship between other Asian population and their pre-pregnancy BMI, suggesting that ethnicity is an independent risk factor modulating insulin resistance among this population(Retnakaran *et al* 2006). In consistency with this finding, level of insulin-sensitizing protein, adiponectin, was found to be decreased in South Asian population(Retnakaran *et al* 2004). Similarly, Moore *et al* observed greater proportion of metformin-treated GDM necessitating additional insulin in Hispanic ethnic group (Moore *et al* 2010; Moore *et al* 2007). At the same time, another study has reported that Hispanics have lower B12 than neighbour matched non-Hispanic White despite being non-vegetarians (Kwan *et al* 2002). Therefore, it could be concluded that relation between ethnicity and insulin resistance might probably be, at least in part, mediated through vitamin B12 deficiency.

1.3.7 Vitamin B12 deficiency and Lipid Metabolism

It has been reported that vitamin B12 deficiency is associated with impaired lipid metabolism. There are also a number of reports from clinical studies that there is significant association between vitamin B12 deficiency and dyslipidaemia (Adaikalakoteswari *et al* 2014). Recently, an adipocyte cell culture study has found that vitamin long-term vitamin B12 deficiency increased intracellular cholesterol biosynthesis by reducing s-adenosylmethionine (AdoMet) to s-adenosylhomocysteine (AdoHcy) ratio and thereby, modifying the expression levels of SREBF1 and LDLR by hypomethylation (Adaikalakoteswari *et al* 2015). They have also reported the negative correlation between serum vitamin B12 levels and total cholesterol, LDL cholesterol and cholesterol to HDL ratio in both pregnant and non-pregnant population (Adaikalakoteswari *et al* 2015). Similarly, the clinical studies looking at vitamin B12 levels and lipid profiles among patients with history of coronary artery disease and type 2 diabetes have reported the same observation that vitamin B12 deficiency was associated with higher triglyceride levels and cholesterol to HDL ratio (Adaikalakoswari *et al* 2014). At the same time, Koplay and his group have also reported that low serum vitamin B12 levels were significantly associated with higher risk of non-alcoholic fatty liver disease and vitamin B12 deficiency could induce high grade liver steatosis (Koplay *et al* 2011). However, there is no molecular study looking at how vitamin B12 deficiency in the liver can give rise to fatty liver disease. Based on the evidence from adipocyte study, one of the possible mechanisms could be through hypo-methylation. A recent study of vitamin B12 supplementation to wistar rats during pregnancy has suggested that there is increased plasma triglyceride levels in offspring of vitamin B12 supplemented rats with decreased eicosapentaenoic acid(EPA) content in the liver (Khaire *et al* 2015). It is reported that EPA regulates triglyceride metabolism by regulating key enzyme involved in triglyceride synthesis.

It is suggested that the pathway could occur through AMPK signalling pathway, which could be another interesting area to explore the mechanism behind how vitamin B12 deficiency contributes to dyslipidaemia.

To explore the possible mechanisms behind, it is first important to understand the regulation of lipid metabolism in the body.

1.4 Lipid metabolism

1.4.1. Transport of Lipids in the body

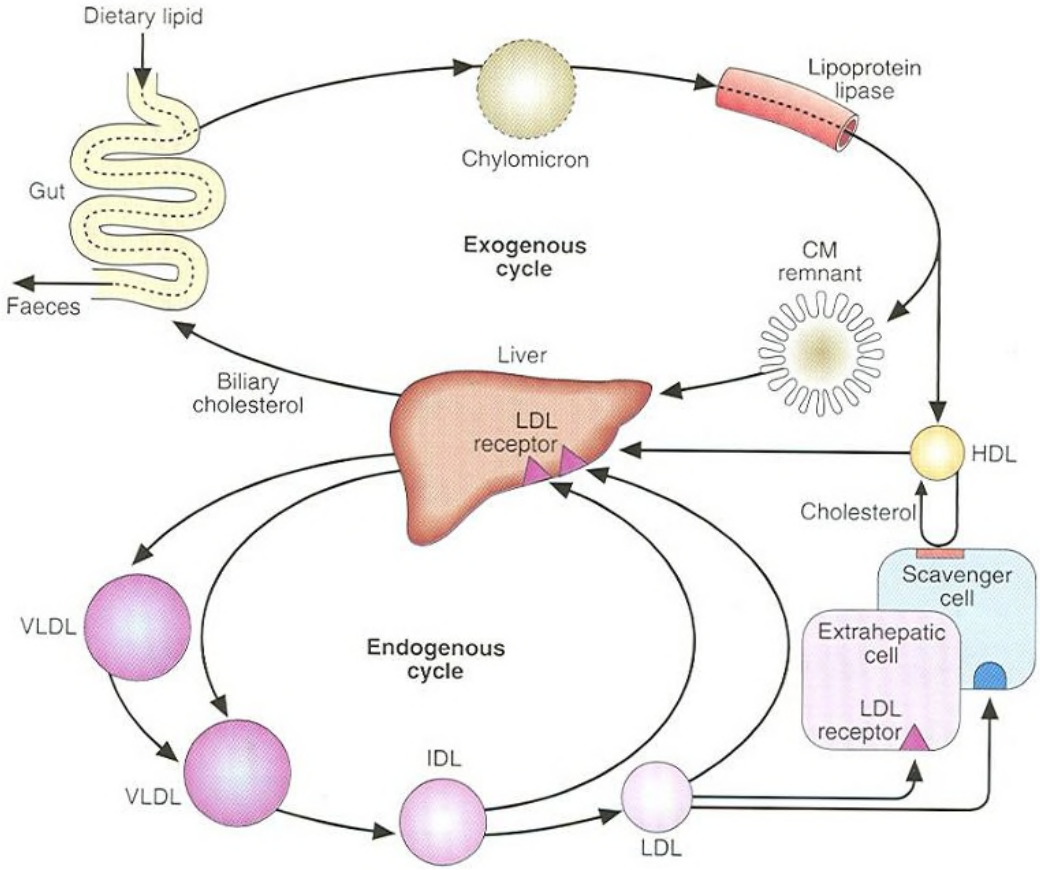
Lipids are important for normal physiologic function in the body not only by serving as energy storage and production but also as cell membrane stabilizer as well as precursors for bile acids and hormones biosynthesis (Nguyen *et al* 2008). Being insoluble in the blood, they are packaged into lipoproteins to travel in the circulation from their site of synthesis or absorption to the sites of utilization. Lipoproteins contain cholesterol ester and triglyceride in the core which are coated by hydrophilic particles such as apolipoproteins (apoB, apoC and apoE), phospholipids and free cholesterol (Kingsbury *et al* 2003). Apolipoproteins serve a number of functions including receptor binding and enzyme activation (e.g. lipoprotein lipase (LPL) by apoC-II which eliminate triglyceride from lipoproteins) and maintaining the structural integrity of lipoprotein particles. There are two main types of lipoprotein in the blood: HDL containing apoA1 and non-HDL containing apoB48 or apoB100. Moreover, depending on sources of lipids, density and lipid components, they can be sub-divided into 5 different classes: chylomicrons, very low density lipoprotein (VLDL), intermediate density lipoprotein(IDL), low density lipoprotein(LDL) and high density lipoprotein (HDL) (Table 1.7). LDL and chylomicrons are main carriers of triglyceride in the blood whereas LDL is major contributor of plasma cholesterol levels (Adeli *et al* 2001; Baltimore *et al* 1999).

Table 1.3. Classification of Lipoprotein in Human Plasma (Baltimore MD Manual of Lipid Disorders: Reducing for Coronary Heart Disease 1999; 2-10.)

Lipoprotein	Major Lipid component	Main Apolipoproteins	Lipid Transport Pathway
Chylomicron	TG	ApoB-48	Exogenous – from intestine to liver
VLDL	TG	ApoB-100	Endogenous – from liver to peripheral tissues
IDL/LDL	CE	ApoB-100	Endogenous – from liver to peripheral tissues
HDL	Protein	ApoA-I	Reverse cholesterol – from peripheral tissues to liver

There are three major lipid transport pathways within the body, namely the exogenous pathway, the endogenous pathway and the pathway of reverse cholesterol transport (Kingsbury *et al* 2003) (Figure 1.8). The exogenous pathway includes dietary lipids which are packaged into chylomicrons by coating with apoB48, produced from intestinal cells, and are carried in the lymphatic circulation to the utilization sites. The endogenous pathway contains lipoproteins produced within the liver. Liver produces triglycerides and cholesterols from plasma non-esterified fatty acid (NEFA) pools and from Acetyl CoA (de novo lipogenesis). Some of them are stored in the liver cytosolic lipid pools whereas some are then packaged with apoB100 to dissolve in the circulation and are carried to the peripheral tissues. The reverse cholesterol transport helps removing cholesterol deposits at the periphery by taking them back in the form of HDL to the liver for clearance. Thus, any defect of the pathways including enzymes like lipoprotein lipase (LPL) – responsible for taking up of triglycerides by peripheral tissue or abnormal apolipoprotein production can result in blood dyslipidaemia(Adeli *et al* 2001).

Figure 1.7. Overview of Lipid Transport in the body (Figure taken from internet available at http://www.clinbiochem.info/studentlipids1.html)



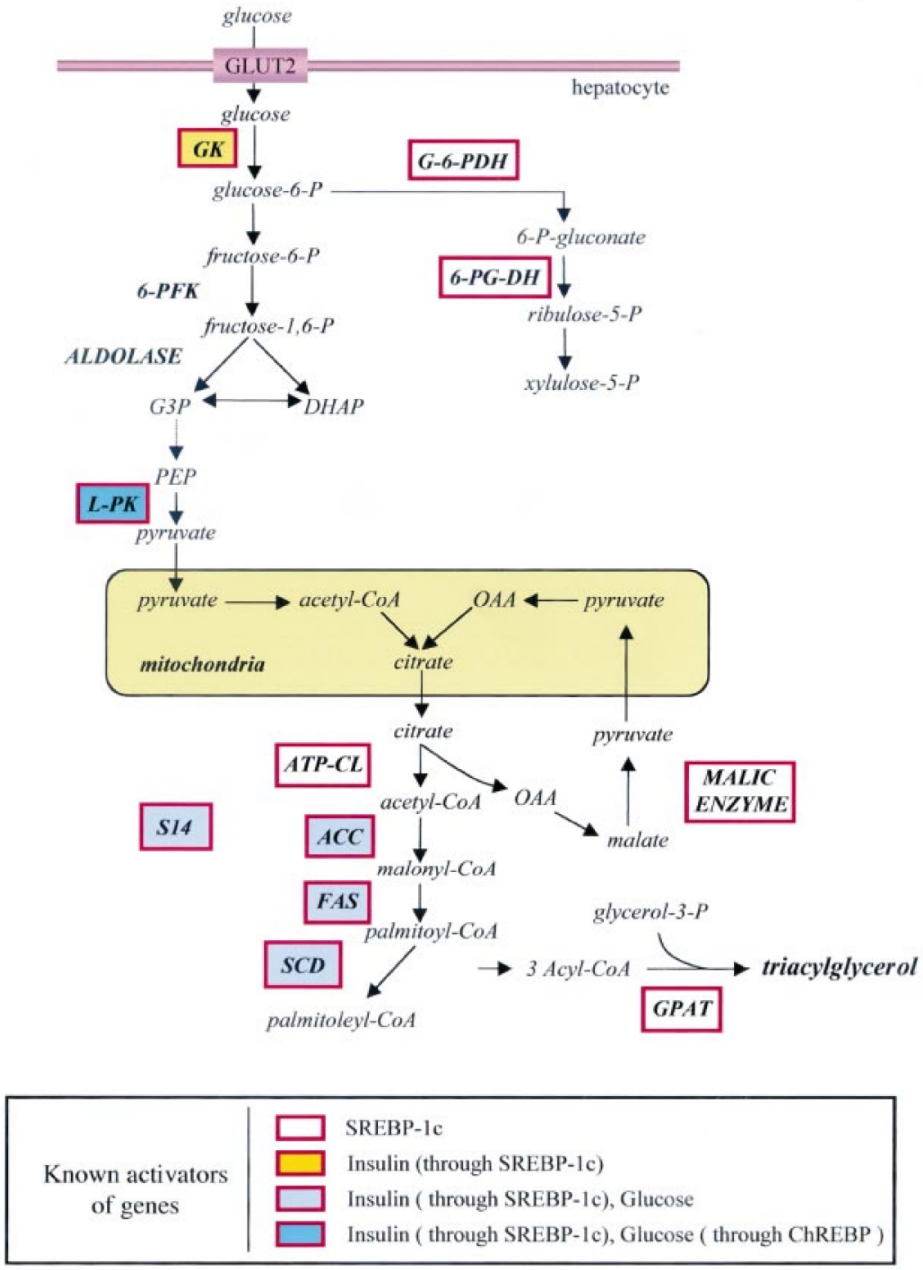
1.4.2. Role of liver in blood lipid regulation

The liver plays a central role in lipid metabolism. The balance between insulin sensitivity and fatty acid availability in the cytosol maintains the intrahepatic lipid composition and, when the influx of lipids are more than the efflux, lipid accumulation in liver (steatosis) develops(Tessari *et al* 2009). The sources of lipids in the liver come from adipose tissue lipolysis contributing to plasma NEFA pools, dietary lipids and de novo lipogenic particles. Their efflux mechanism includes oxidation and VLDL secretion.

1.4.2.1.Liposynthesis, *de novo* lipogenesis and VLDL assembly and secretion

Liver takes up triglycerides in the circulation via hydrolysis by hepatic lipase (HL) and LPL enzymes whereas LDL cholesterol remnants are endocytosed by specific LDL receptor (LDLR) on the surface (Nguyen *et al* 2008). Free fatty acids (FFA) (activated as fatty acyl CoA) from NEFA pools are esterified with glycerol (activated as alpha-glycerophosphate [α-GP]) by glycerophosphate acyltransferase (GPAT). GPAT gene transcription and activity are stimulated by insulin and nutritional status, while glucagon inhibits it. The esterification and lipoprotein synthesis are activated by FFA flux to the liver. Moreover, the internalized LDL cholesterol are hydrolysed in the lysosomal enzymes for further usage in bile acid production and membrane phospholipid synthesis.

Figure 1.8. An illustrative model of hepatic glycolytic and lipid pathways. All the enzymes in the figure are induced at transcriptional levels by a high-carbohydrate diet through ChREBP and SREBP transcription factors (the known transcription factors and their regulated genes are described at the bottom). (Figure taken from Foufelle F *et al*. Biochem J 2002 Sep 1; 366(Pt2):377-91)

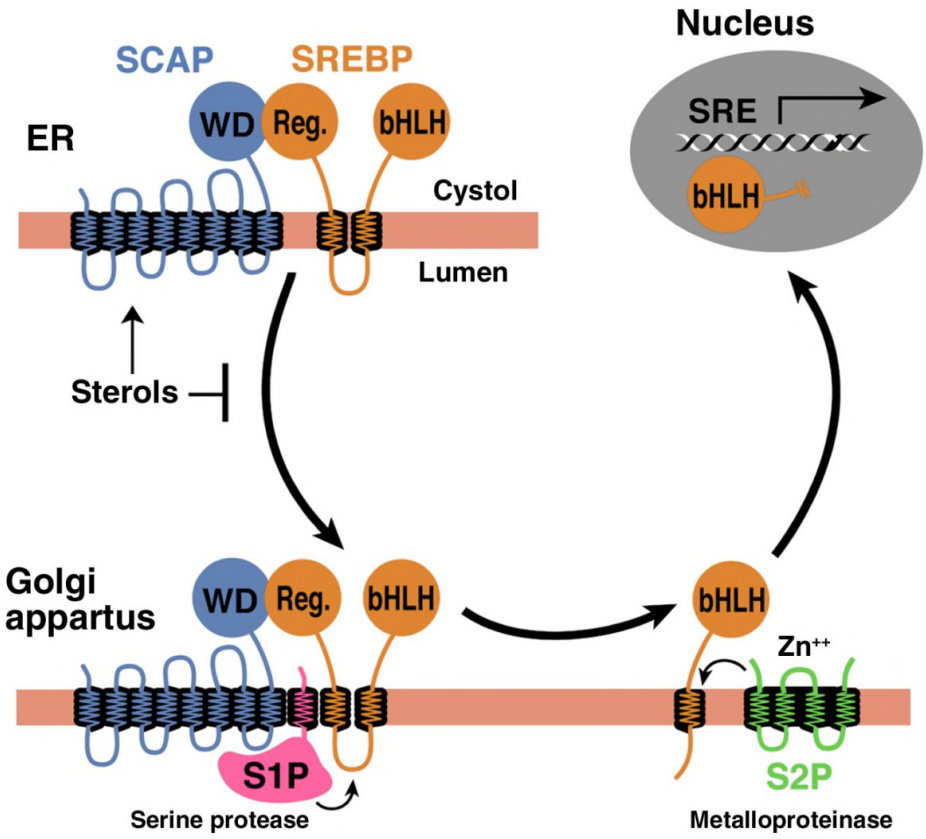


De novo lipogenesis

In humans, liver is the major organ for *de novo* lipogenesis (Kersten 2001). Glucose enters into hepatocyte by the help of facilitated glucose transporter (GLUT2) which is a high capacity low affinity glucose transporter. Once in the cell, it is converted to glucose-6-phosphate (G6P) by glucokinase(GK), which is the rate-limiting enzyme for further glucose utilization (Figure 1.9). Then, G6P binds to carbohydrate responsive element binding protein (ChREBP) which regulates genes involved in glycolytic and lipogenic pathways such as pyruvate kinase(PK), FAS and ACC by binding to glucose or carbohydrate-response elements(ChoRE) on the promotor region of the target genes. ChREBP is also an essential link between carbohydrate and lipid metabolism (Raddatz *et al* 2011). Sterol regulatory element binding factor/protein-1c (SREBF1c/SREBP1c) is also another transcriptional factor which regulates triglyceride synthesis and capacity of storage inside the liver (Horton *et al* 2002). SREBP are normally resided in endoplasmic reticulum (ER) and they control fatty acid metabolism by integration of signals from different pathways (Figure 1.10). They are stimulated when intracellular lipid levels are low (SREBP1c and SREBP2 preferentially for triglyceride and cholesterol biosynthesis respectively). Low cholesterol levels are initially sensed by SREBP cleavage-activating protein (SCAP) which subsequently transports SREBP from ER to Golgi apparatus. The SERBP/SCAP complex is then cleaved by two proteases, Site-1 protease (S1P) and Site-2 protease (S2P), releasing NH_2-terminal domain of basic helix-loop-helix-leucine (bHLH-Zip) region from the membrane. The newly synthesized bHLH-Zip domain translocates to the nucleus where it binds to sterol response elements (SREs) in the promotor regions of various target lipogenic enzymes including PK, FAS, ACC, Stearyl-CoA (SCD) and 3-Hydroxy-3-Methylglutaryl-CoA Reductase (HMGCR).

Apart from GK which is only under insulin control, all other genes involved in glycolytic and lipogenic pathways can be induced by both insulin and glucose (Raddatz *et al* 2011). As mentioned above, insulin enhances *de novo* lipogenesis by activation of glucokinase and SREBPs. Of these, ACC and HMGCR are rate-limiting enzymes in triglyceride and cholesterol synthesis respectively (Nguyen *et al* 2008).

Figure 1.9. An illustration of how SREBPs are released from endoplasmic reticulum(ER). (Taken from Horton JD. J. Clin Invest. 2002. 109:1125-1131)



De novo lipogenesis is initiated with acetyl CoA formation from pyruvate by L-PK(Foufelle *et al* 2002). Then, under the insulin control, acetyl CoA is carboxylated to malonyl CoA which further undergoes triglyceride synthesis while it is converted to Hydroxy-3-Methylglutaryl-CoA (HMG-CoA) by Hydroxy-3-Methylglutaryl-CoA Synthase (HMGCR), both of which are in independent of FFA (Figure 1.9). ACC is bidirectional enzyme which regulates FFA partitioning between oxidation and lipogenesis at the availability of malonyl CoA. Under the insulin control, malonyl CoA accumulation favours FFA towards esterification and triglyceride production while inhibiting fatty acid beta-oxidation (β-oxidation) by blocking the activity of mitochondrial fatty acyl transporter, carnitine palmitoyltransferase-1 (CPT-1)(Kersten *et al* 2001). By contrast, glucagon enhances β-oxidation and ketogenesis by stimulating CPT-1 activity. On the other hand, the overflow of FFA to liver, e.g. diabetic ketoacidosis, can saturate both pathways denoted by combined hyperlipidaemia and hyperkitonemia. After esterification, the newly synthesized FFA molecule are elongated by FAS and the resultant palmitoyl-CoA is desaturated by SCD to palmitoleyl CoA, both of which are under insulin control(Nguyen *et al* 2008). At the same time, HMG-CoA in cholesterol biosynthetic pathway is further reduced by HMGCR, forming mevalonate. Mevalonate is then eventually converted to cholesterol by the action of farnesyl diphosphate synthase and squalene synthase. All the enzymes described above in cholesterol biosynthesis are under SREBP2 control. Interestingly, the SERBPs stimulation by insulin can be observed in states of marked insulin resistance and thereby favouring de novo lipogenesis(Foufelle *et al* 2002; Adiels *et al* 2008). The other transcription factors involved in de novo lipogenesis includes peroxisome proliferator-activated receptors-gamma (PPAR-γ) stimulated by SREBP-1c activation and newly discovered regulatory protein Spot 14 (S14) stimulated by

high-carbohydrate diet and thyroid hormone. Interestingly, it has been reported that only a small part of total triglyceride synthesis in liver is affected by de novo lipogenesis in humans (Postic *et al* 2008). However, whether this pathway is profoundly affected in the state of insulin resistance is still an area required for further exploration.

1.4.2.2.Lipid transport

The newly synthesized lipids are then exported out of liver by binding to ApoB100 (Adeli *et al* 2001). Increased FFA and TG levels and mitochondrial transfer protein (MTP) stimulate ApoB biosynthesis whereas insulin inhibit it. MTP initiates lipidation of nascent apoB molecule and further enhances the assembly of apo-B containing lipoproteins by catalysing the transfer of lipids from triglyceride rich droplets in smooth endoplasmic reticulum to nascent Apo-B particle.

1.4.2.3. Lipid oxidation

The oxidation of non-esterified acyl-CoA occurs in mitochondria, peroxisomes and microsomes in liver (Nguyen *et al* 2008). Oxidation of very long chain fatty acids occur in peroxisome and microsomes whereas mitochondria is responsible for oxidation of short, intermediate and long fatty acids. The lipid oxidation is regulated in the liver by the balance between fatty acid supply (from lipolysis of adipocytes) and the rate of lipid oxidation and esterification by microsomes.

Intramitochondrial oxidation of fatty acids with more than 14 carbon requires carnitine parmitoyl transferase I (CPT-I) enzyme because mitochondrial matrix lack the Acetyl-CoA synthetase (ACS) enzyme responsible for activation of these fatty acids. CPT-I is again negatively controlled by mitochondrial malonyl-CoA levels, which is the first

intermediate of de novo lipogenesis, catalyzed by acetyl coA carboxylase (ACC) under the control of insulin (Postic *et al* 2008). When there is intracellular negative energy balance and low malonyl CoA levels, fatty acyl CoA from plasma non-esterified fatty acid pools are carried into mitochondria with the help of the transporter, CPT II, for beta-oxidation. Once in mitochondria, they are converted to malonyl CoA and then to succinyl CoA, an essential intermediate of krebs citric acid (KCA) energy cycle. Finally, succinyl CoA enters into the KCA cycle and energy is released. CPT-1 can also be regulated by methylmalonyl CoA(MM CoA) which is the product of odd chain fatty acid oxidation and requires vitamin B12 for conversion to succinyl CoA(Nguyen *et al* 2008). Thus, in case of vitamin B12 deficiency, MM CoA is accumulated and inhibits CPT-1 and impairs fatty acid oxidation.

Peroxisomal and microsomal fatty acid oxidation is much similar to mitochondrial oxidation(Nguyen *et al* 2008).However, they produces less ATP-energy than mitochondria because they do not have electron transport chain. Microsomes have cytochrome P450 CYP4A ω-oxidation system which allows them to hydrolyse very long chain fatty acids into much shorter fatty acids which are preferred substrate for peroxisomal oxidation. Then, the products of peroxisomal oxidation enter into mitochondria for further oxidation. When there is fatty acid overload, these three systems work co-ordinately to regulate fatty acid metabolism inside the liver.

1.4.2.4.Hepatic glucose output

Liver also involves in glucose production by glycogenolysis and gluconeogenesis from non-carbohydrate precursors such as lactate and glycerol(Raddatz *et al* 2011). Glucose comes mainly from glycogenolysis within 2-6 hours of meal while gluconeogenesis mainly contributes to blood glucose levels during prolonged fasting.

The enzymes controlled gluconeogenesis are phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-biphosphatase(FP2ase) and glucose-6-phosphatase(G6Pase) which are inhibited by insulin and stimulated by glucagon or glucocorticoids.

1.4.2.5.Insulin signalling

Insulin signalling is initiated by binding of insulin to its cell surface receptor (Kim *et al* 2010). The insulin receptor complex then activates tyrosine kinase activity which phosphorylates subsequent downstream molecule, insulin receptor substrates (IRSs), such as IRS-1 and IRS-2. The stimulated IRSs lead to the docking of phosphoinositol-3 kinase (PI-3K) (p85-p110) which further phosphorylates Akt. The phosphorylated Akt subsequently phosphorylates and inhibits the activity of glycogen synthase kinase (GSK) which blocks glycogen synthase for glycogen synthesis.

As we have described above, normal liver function is very important for lipid metabolism as it plays central role in the regulation of blood lipid levels. At the same time, vitamin B12 deficiency has possibility of contribution to insulin resistance and blood dyslipidaemia. There are very limited number of studies looking at mechanism behind vitamin B12 deficiency and abnormal lipid metabolism. It would be of great value if the possible cellular mechanism of vitamin B12 deficiency is fully established in the development of vitamin B12 supplementation strategy.

In summary, the role of metformin in GDM is increasing. However, metformin can reduce vitamin B12 levels, even given for short term period of metformin administration. Vitamin B12 is an essential micronutrients during pregnancy and it functions in close relationship with folates. Moreover, there is evidence that vitamin B12 deficiency can induce insulin resistance and dyslipidaemia. Thus, it was

hypothesized that if vitamin B12 is deficient in the cell, metformin cannot function at its full potential.

1.5 Thesis Aims and methods

The thesis aims to explore factors influencing the efficacy of metformin in the treatment of GDM and the role of micronutrients, particularly vitamin B12, in treatment failure. It comprised of a systematic review, clinical and cell culture studies to evaluate role of metformin in GDM, factors influencing its efficacy and mechanisms by which vitamin B12 deficiency interferes with metformin study.

Systematic review

- To evaluate efficacy and safety of metformin in the treatment of GDM

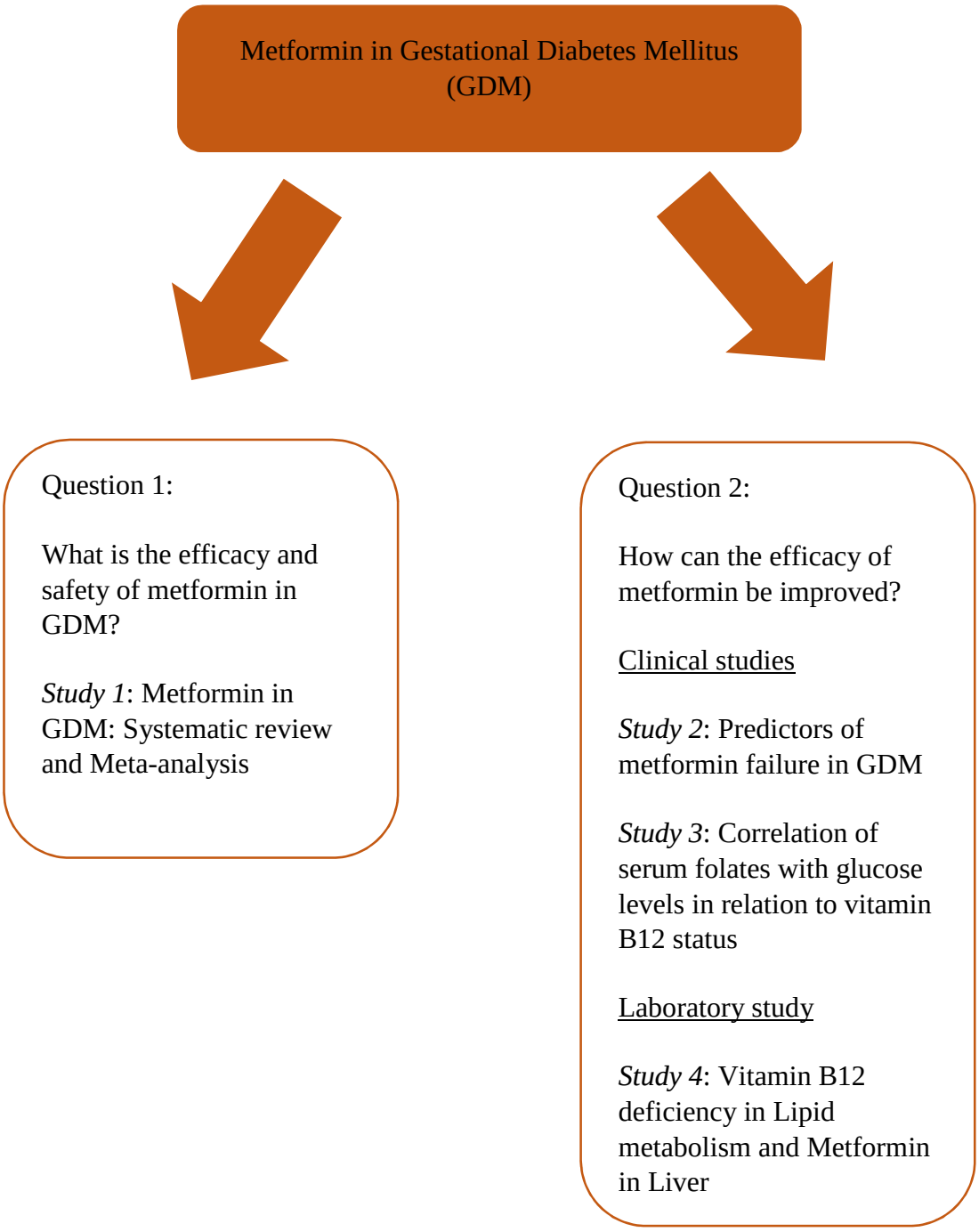
Clinical studies

- To identify maternal characters of metformin failure in GDM
- To investigate the association between vitamin B12, folate & homocysteine with the pregnancy outcomes in GDM

Laboratory study

- To explore the mechanisms by which B12 deficiency interfere with metformin action in hepatocytes

Figure 1.10. An overview of PhD thesis



CHAPTER 2

EVALUATION OF METFORMIN IN GESTATIONAL DIABETES: SYSTEMATIC REVIEW AND META-ANALYSIS

(STUDY 1)

2.1 Introduction

GDM is the commonest medical disorder in pregnancy and the prevalence would be tripled if the new IADPSG guidelines are introduced and can be up to 25%(Duran *et al* 2014; Cundy *et al* 2014). Moreover, the prevalence of GDM women has also been increased by 64% in the past 2 decades, most possibly due to increasing maternal obesity and aging mothers at first pregnancy (Duran *et al* 2014). Thus, it could be well-expected that the percentages of GDM will be booming up in next couple of years. Currently, clinical emphasis is on the achievement and maintenance of maternal glycemic control, which acts as a modifiable principal predictor of adverse outcomes to mother and fetus (Rowan *et al* 2010). Importantly, hyperglycemia during pregnancy is strongly associated with neonatal hypoglycemia and impaired foetal lung maturity leading to respiratory distress and polycythemia after birth (Langer *et al* 2008). GDM also has a negative impact on the risk of metabolic diseases and impaired cognitive function in later life(Langer *et al* 2008; Clausen *et al* 2008; Pirkola *et al* 2010). Management of GDM starts with lifestyle modification followed by medical intervention. Almost half of GDM women need medication which include insulin and oral hypoglycaemic agents because of poor glycaemic control (Terttie *et al* 2008; Goh *et al* 2011). The role of oral hypoglycaemic agents in GDM is increasing nowadays. Metformin and glibenclamide are now approved to be offered to GDM women in some guidelines,but their efficacy and safety are not well-understood yet. Considering the strong association between GDM and obesity, metformin is a promising drug for GDM because it can benefit on lowering maternal weight gain than insulin.

Up to date, there are six systematic reviews which have looked at the evidence of metformin in gestational diabetes compared to insulin and glibenclamide (Balsells *et*

al 2015, Kitwitee *et al* 2015; Su *et al* 2014; Gui *et al* 2013; Lautatzis *et al* 2013; Amin *et al* 2015). The most recent one included 8 randomized controlled trials (RCTs) in their outcome estimate synthesis(Kitwitee *et al* 2015). Although metformin may benefit on GDM than either insulin or glibenclamide, the effects are still varied and there are large heterogeneity among studies. This might be due to differences in diagnosis of GDM (e.g.75g or 100g OGTT) or variations in intervention strategies. Moreover, it is reported that metformin use in GDM is associated with higher incidence of prematurity (Rowan *et al* 2008). Inclusion of non-randomized controlled trials (NRCT) will increase the sample size and will also enable subgroup analysis and sensitivity analysis to generate more specific and precise outcome estimate with wider inferential value. Furthermore, as these NRCTs have much more clinically pragmatic design (i.e. inclusion of physician's judgement on treatment therapy), this might also help research methodology to be developed in future studies.

At the same time, another concern for metformin in GDM is that up to 50% of women required additional insulin treatment. Exploration of the factors that can predict GDM subgroup with high potential of supplementary insulin will allow avoidance dose titration period with metformin which usually takes around 1 to 3 weeks and help better glycaemic control from the time of GDM diagnosis until delivery. Thus, it has been carried out the systematic evaluation of current available evidence of metformin in GDM to fully appreciate its efficacy, limitation and implacability and to explore the maternal characters that can predict metformin failure.

2.2 Methodology

This review was conducted after a review protocol had been developed with general agreement on the objectives and methodology among 4 reviewers [May Oo Khin (MOK), Manu Vatish (MV), Simon Gates (SG), Ponnusamy Saravanan (PS)].

2.2.1 Criteria for considering studies for this review

i) Type of studies

All published observational studies or randomized trials comparing metformin with other blood glucose control alternatives were included. For metformin failure studies, metformin alone compared with metformin plus insulin (metformin failure group) were considered to include in this review. Only studies with primary data were considered, i.e, compilation of cases from hospital data or case note reviews were excluded because of the concerns over validity of the findings and lack of comparisons.

ii) Type of participants

Pregnant women with any degree of glucose intolerance must be identified by standard 75 (or) 100 grams oral glucose tolerance test (OGTT). Gestational age at diagnosis of GDM must be after 10 weeks. Studies of women with pre-pregnancy diabetes (type 1 or 2) were excluded. Studies ≥ 80% GDM population in each arm or those with separate analysis of GDM for outcomes considered were included in this review. GDM was defined as fasting glucose level ranging from 4.8 mmol/l (86 mg%) to 7 mmol/l (126 mg%) or 2hr glucose level regardless of 75 or 100g OGTT between 5.6 mmol/l (101 mg%) to 11 mmol/l (198 mg%) of which upper limits were regarded as impaired fasting glucose or impaired glucose tolerance in type 2 diabetes population.

iii) Type of intervention

Metformin or comparator treatment (insulin, glibenclamide) must be first-line treatment for women with GDM who failed to achieve target glucose level with lifestyle changes (nutritional therapy and/or exercise). However, the outcomes of metformin group compared to dietary intervention alone were also considered if they were reported separately. For metformin failure studies, if there were separate report of metformin and metformin failure groups on maternal characters and pregnancy outcomes, they were considered eligible. Moreover, metformin must be introduced only after diagnosis of GDM with the aim of achieving glycemic control. Studies with co-intervention or mixed intervention using other oral hypoglycemic agents without separate analysis of metformin were not considered. Trials with metformin compared to lifestyle intervention or insulin or any other oral hypoglycemic agents were included. Interventions with metformin using insulin either at baseline or after metformin failed to achieve glycemic control (metformin failure) were also included.

iv) Type of outcome measures

Any primary outcome was not specified. But, the specific focus was on important outcome measures relevant to sequales of gestational diabetes and potential side-effects of metformin.

Important clinical outcomes and their clinical definition

1. Foetal birth weight (BWt) in grams
2. Macrosomia (BWt> 4000g)
3. Large for gestational age (LGA) > 90 percentiles for gestational age
4. Small for gestational age (SGA) > 10 percentiles for gestational age
5. Stillbirth/peri-natal mortality rate(intrauterine foetal death after 20 weeks gestation)
6. Birth trauma
7. Shoulder dystocia
8. Caesarean section rates instrumental delivery
9. Neonatal respiratory distress
10. Gestational age at delivery
11. Premature baby(<37 weeks gestation)
12. Neonatal hypoglycemia
13. Pre-eclampsia
14. Induction of labour
15. Neonatal jaundice
16. Admission to special care baby unit(SCBU)/neonatal intensive care unit(NICU)
17. Maternal fasting glycaemic control during pregnancy

18. Maternal postprandial glycaemic control during pregnancy
19. 5 minutes APGAR score <7
20. Arthropometric measures at birth
21. Maternal weight gain

The following outcomes were also described although they were only relevant to metformin

1. GDM with tolerability of treatment
2. Metformin-treated GDM with additional insulin
3. Risk of any side-effects necessitating action

2.2.2 Search methods for identification of studies

Depending on the objectives of systematic review, the search was devised into a series of search concepts.

The main concepts used were “metformin”, “gestational diabetes” and “impaired blood glucose” during “pregnancy”. We did not restrict the search to the outcomes to be as inclusive as possible. Studies included in this review must be the primary studies. Alternative terms for the main 3 concepts were thoroughly searched and applied. Search terms tabulated as follows:

Table 2.1 Initial search terms used

1.Health condition	gestational diabetes, glucose intolerance, abnormal blood/plasma glucose, impaired blood glucose/glucose level/glucose control, abnormal blood sugar level/control, blood glucose or blood sugar impairment, insulin resistance, hyperglycemia, pregnancy-related/induced diabetes
2.Population	pregnancy, second trimester, third trimester, late pregnancy
3.Intervention	metformin, glucophage, diabex, gliphage, glafornil, merckfomin, risidon, dianben, dimethylbiguanide, dimethylbiguanidium, dimethylguanylguanidine, glumetza, fortamet, glycomet, diaformin, diformin, bolamyn, riomet
4.Design	No restriction of study design for sensitivity rather than precision

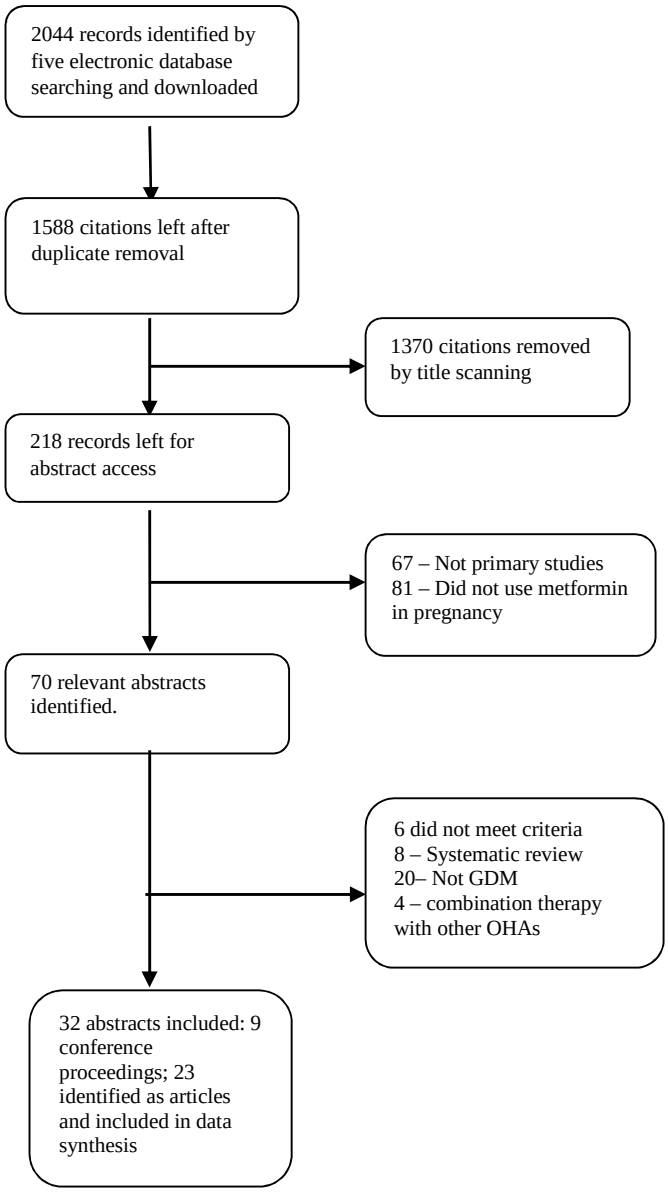
The search terms were applied in 5 electronic databases (Ovid MEDLINE, EmbaseClassic+Embase1947 onwards, Cochrane collaboration resources, The Nursing & Allied Health (CINAHL) database, Web of Science). Ongoing trials were also searched in the ISRCT Registry (www.controlled-trials.com). Medical

Subject Heading (MeSH) searches were applied for each general search term and the initial results of each search term including alternatives were combined using Boolean search terms ‘AND’ or ‘OR’ as appropriate. The details strategies of search term applied to each database were described in Appendix 9.1.1. No restriction to language, country and date was applied. Moreover, reference lists of related systematic reviews and included studies were also hand-searched. Finally, electronic email updates were registered. The search strategy was last updated in May, 2015.

2.2.3 Selection of studies

Initially, screening of relevant studies was done by scanning the titles in each database. At this stage, a low threshold of inclusion criteria was applied with the aim of high sensitivity for detected citations, i.e. titles stating the use of metformin during diabetic pregnancies were all included if they seemed to be primary studies or reviews. Then, the results were downloaded to the bibliographic database software, Endnote. Following duplicate removal, abstracts were scanned and potentially relevant citations were grouped into primary studies. Then, the eligibility criteria were applied to the included primary studies, and the references of second-stage included primary studies and systematic reviews were hand searched for other relevant citations. The details of the flow of study selection were described in figure 2.1.

Figure 2.1: PRISMA flow chart of study selection



Of the 1607 identified articles, 1370 did not match our selection criteria based on review of their titles conducted by two authors (MOK & MV). After abstract scanning, 148 records were removed and the reasons for exclusion were that the articles were not primary studies (67 studies) and did not use metformin in pregnancy (81 studies). These two authors then independently reviewed the full text

of the remaining 70 articles to determine inclusion or exclusion (Figure 2.1). When duplicate data were identified, only the most up-to-date, larger series was included. Of the remaining 70 citations, 23 published articles were deemed eligible for inclusion. These articles consisted of 12 individual Randomised Controlled Trials (RCTs) of metformin in GDM compared to insulin (with three continuation report)(Moore *et al* 2007; Rowan *et al* 2008; Rowan *et al* 2011; Ijas *et al* 2010; Ijas *et al* 2015; Hassan *et al* 2012; Niromanesh *et al* 2012; Spaulonci *et al* 2013; Mesdaghinia *et al* 2013; Tertti *et al* 2013; Ruholamin *et al* 2014; Battin *et al* 2015) and 8 individual Non RCTs (Tertti *et al* 2008; Balani *et al* 2009; Rai *et al* 2009, Goh *et al* 2011; Gandhi *et al* 2012; Iftakhar *et al* 2012), 3RCTs studies compared to glibenclamide (Moore *et al* 2010; Silva *et al* 2013; George *et al* 2015), one study compared to di*et al*one (Balani *et al* 2012)and one study for predictor of metformin failure (Corbould *et al* 2013). Any disagreements about study eligibility were resolved by consensus, with arbitration by the other authors (SG & PS) if necessary.

2.2.3 Data Extraction and analysis

Data were extracted using a predefined data extraction proforma sheet with explicit instructions (Appendix 9.1.2). The main data collected were methods of selection and characteristics of participants potentially related to baseline comparability, detailed intervention carried out for assessment of performance bias, outcome measures with explicit definition for comparability in-between studies and for assessment of detection bias and any statistical analysis including subgroup analysis. The study conclusion, ethical approval, funding and any conflict of interest were also extracted. An attempt to obtain missing data was done and included in this review. The outcomes from duplications of the primary studies were

also examined and presented(Silva *et al* 2010; Silva *et al* 2013). Pilot testing of the data proforma sheet and risk of bias tool were performed on a few studies (2 observational and 2 randomized studies) by the first reviewer (MOK) and checked by the second (MV) and third reviewers (PS). Then, the agreed sheets were used by the first reviewer to apply on remaining studies. Any disagreement was resolved by discussion. No significant changes were made to the proforma sheet and bias tool.

2.2.4 Assessment of quality of included studies

The general and specific bias items were assessed using pre-specified agreed risks of bias tools with explicit criteria(Appendix 2.2) separately for RCTs and NRCTs, created based on PRISMA statement (Liberati *et al* 2009) and Cochrane handbook (Higgins *et al* 2011). The general categories of items assessed for internal validity for both randomized controlled trials (RCTs) and non-randomized controlled trials (NRCTs) were on selection of population (eligibility, inclusion and exclusion criteria and general clinical management before metformin was given), performance of clinical care (comparable clinical monitoring, follow-up and delivery plan after medication was initiated until delivery), detection of outcomes (blinding of outcome assessors), intention-to-treat anlaysis/ attrition of samples (analysis of all GDM treated with specified medication within study time frame), number of outcomes reported (inclusion of all the important outcomes specified in this review) and other possible biases (e.g. interim analysis). For external validity, separate items on population, intervention and explicit outcomes were justified to examine how transparent in information for replicability and comparability across studies. Basically, studies were assessed with careful consideration of reporting important issue by each author.

2.2.5 Data synthesis

Raw data were tabulated to illustrate the differences in population selection, intervention undertaken and other design features that varied between studies. The risk of bias tables were also created to compare the heterogeneity in between studies for reliability of assumptions of outcome estimates for this review. When clinically and methodologically appropriate, RevMan 5.1 meta-analysis software was used to pool the results separately depending on their study design. Chi-squared test was used to detect the heterogeneity between studies and p value was used to determine the statistical significance. The outcome estimates of RCTs and NRCTs were combined separately using a random effect meta-analysis model. I^2 was used to quantify the inconsistency. If $I^2 \geq 20\%$, individual clinical data and methodological features on each included study were carefully accessed to determine the source of the heterogeneity. The subgroup analysis was done and reported separately according to 75g or 100g OGTT diagnosis test if there were adequate number of studies. Differences in GDM diagnosis, criteria for medical intervention and brief overview among the studies were compared and tabulated. A table was also constructed to compare the differences in studies, published in conference proceedings.

2.3 Results

The diagnostic criteria and target glucose levels among 20 different studies were described in table 2.2 and 2.3.

Table 2.2: Criteria for diagnosis of GDM

Study ID	1step or 2 steps	OGTT	Number needed to be abnormal		FBS mmol/l (mg/dl)	1hr mmol/l (mg/dl)	2hr mmol/l (mg/dl)	3hr mmol/l (mg/dl)
Balani 2009	1	75 g	≥1				≥7.8	
Balani 2012	1	75g	≥1		>6		>7.8	
Corbould 2013	2	75g	≥1		>6		>7.8	
George 2015	1	100g	≥2		≥5.3	≥10	≥8.6	≥7.8
Goh 2011	2	75 g	≥1		≥5.5		≥9	
Gandhi 2012	1	75 g	≥1		>6		≥7.8	
Hassan 2012	2	75 g	≥2 out of 3		>5.3	≥10	≥8.6	
Iftakhar 2012	1	75 g	≥1	insulin	>6		≥9	
				metformin	>6		≥7.8	
Ijas2010	1	75g	≥1		>5.3	>11	>9.6	
Moore 2007	2	100 g	≥2		>5.8(105)	>10.6(190)	>9.2	>8.1
Moore 2010	2	100 g	≥2		>5.3	>10	>8.6	>7.8
Mesdaghinia 2013	2	100 g (2 step)	≥2		> 5.3(95)	>10(180)	> 8.6 (155)	> 7.8 (140)
Niromanesh 2012	2	100 g	≥2		≥5.3(95)	≥10(180)	≥8.6 (155)	≥7.8 (140)
Rowan2008	1	75 g	≥1		>5.5		>8(Aus) >9(NZ)	
Rai 2009	2	100 g	≥2		≥5.3 (95)	≥10 (180)	≥8.6 (155)	≥7.8 (140)
Ruholamin 2014	1	75g	≥1		5.1	10	8.5	
Silva 2012	1/2	75 g 100 g	≥2		≥7 ≥5.3	 ≥10	≥11.1 ≥8.6	 ≥7.8
Spaulonci 2013	1	75/100 g	≥2		≥5.3(95)	≥10(180)	≥8.6 (155)	≥7.8 (140)
Tertti 2008	1	75g	2 out of 3		≥4.8	≥10	≥8.7	
Tertti 2013 2013(2006-08) 2008-2010	1	 75g 75 g	2 out of 3 2 out of 3		 ≥4.8 ≥5.3	 ≥10 ≥10	 ≥8.7 ≥8.6	

1 step – Screened by demographic high risk factors followed by 75/100g OGTT
2 steps – Screened by 50g Oral Glucose Challenge Test (OGCT) followed by 75/100g OGTT

Table 2.3 : Criteria for starting medical intervention

Study ID	Number needed to be abnormal/ 1-2weeks	Fasting Glucose mmol/L (mmol/L)	Postprandial Glucose mmol/l(mg/dl)		Maximum Metformin dose (mg/day)	Metformin Failure rate (%)
			Timing	Abnormal		
Balani 2009	≥3	≥6	1hr 2hr	≥8 ≥7	2500	10.2
Balani 2012	≥3	>5.6	1hr 2hr	>8 >7	3000	11.7
Corbould 2013	≥3	>5.6	1hr 2hr	>8 >7	2000®	30.4
George 2015	≥1	≥5.5 and ≤7.2	2hr	≥6.7 and ≤13.9	2500	0
Goh 2011	≥1	>5	2hr	>6/6.5	2500 (3000 for morbidly obese)	46.5
Gandhi 2012	≥1	>6	1hr	>8	2000	21
Hassan 2012	≥1	≥5.5	1.5hr	≥7	3000	24
Iftakhar 2012	≥3	≥5.5	2hr	≥7	2000	41.8
Ijas2010	≥1	≥5.3	1.5hr	≥6.7	2350®	31.9
Moore 2007	≥1	≥5.8 (105)	2hr	≥6.7 (120)	2000	0
Moore 2010	≥2	>5.8 (105)	2hr	>6.7 (120)	2000	34.7
Mesdaghinia 2013	≥1	≥5.28(95)	2hr	≥6.67(120)	2500	22
Niromanesh 2012	≥2	≥5.3(95)	2 hr	≥6.7(120)	2500	14
Rowan2008	≥1	>5.4	2hr	>6.7	2500	46.3
Rai 2009	≥2	≥5.6 (100)	2hr	≥7.2 (130)	2000	7
Ruholamin 2014	≥2	≥5.28(95)	2hr	≥6.67(120)	1500	3
Silva 2012	≥2	>5 (90)	1hr	>6.7 (120)	2500	21
Spaulonci 2013	>30%	>5.3(95)	2 hr	>6.7(120)	2550	26.08
Tertti 2008	≥2	≥5.5	1.5hr	≥7.8	1500	18
Tertti 2013	≥1	≥5.5	1 hr	≥7.8	2000®	20.9

® = Extended-release metformin

2.3.1. Comparison 1: Metformin vs Insulin

Table 2.4. Quality assessment of included RCTs studies (Metformin vs Insulin)

Study	Internal Validity										External validity				Potential bias
	Selection		Performance		Detection	Attrition			Reporting	Others					
	Randomization	Allocation concealment	Double Blinding	Same Care pathway	Blinding of outcome assessors	Prior sample size	Intention-to-treat analysis	Acceptable attrition rate	Comprehensive outcome report	Free from other risks of bias	Population	Intervention	Comparator	Explicit reported outcomes	
Moore 2007	+	+	-	+	-	+	+	-	-	-	+	+	+	-	Moderate
Rowan2008	+	+	-	+	+	+	+	+	+	-	+	+	-	+	Low
Ijas2010	+	?	-	+	-	+	-	-	-	-	+	+	-	+	High
Hassan 2012	-	-	-	+	-	-	?	?	-	+	+	+	-	+	High
Niromanesh 2012	+	+	?	+	+	+	+	+	+	-	+	+	+	+	Low
Spaulonci 2013	?	-	-	-	-	+	+	+	-	+	+	+	+	-	High
Mesdaghinia 2013	+	+	?	+	+	+	-	?	-	+	+	+	+	-	Moderate
Tertti 2013	-	?	-	-	-	+	-	-	-	?	+	+	-	+	Low
Ruholamin 2014	+	-	?	+	+	+	-	-	-	+	+	+	+	+	Moderate

2.3.1.1. Quality assessment of included studies compared to insulin (Table 2.4)

2.3.1.1.1 Randomized studies

Randomization and allocation concealment was adequately performed in 4 out of 9 RCTs (Moore *et al* 2007; Rowan *et al* 2008; Niromanesh *et al* 2012; Mesdaghinia *et al* 2013). Although 3 studies were reported to perform double-blinded, they did not mention in details. As insulin and metformin have different routes of administration, it should be reported in details about how the intervention was blinded from participants (Niromanesh *et al* 2012; Mesdaghinia *et al* 2013; Ruholamin *et al* 2014). The RCTs done by Spaulonci 2013 and Ruholamin 2014 excluded metformin failure group from metformin arm and thus the same baseline comparability should be suspected. Only 4 out of 9 RCTs blinded outcome assessors from treatment(Rowan *et al* 2008; Niromanesh *et al* 2012; Mesdaghinia *et al* 2013; Ruholamin *et al* 2014). Although sample size was determined in 8 studies, the appropriate case analysis was performed in only 3 studies (Rowan *et al* 2008; Niromanesh *et al* 2012; Spaulonci *et al* 2013). The important clinical outcomes of GDM considered in this review were reported completely by Rowan 2008 and Niromanesh *et al* 2012. In addition, three studies did interim analysis(Moore *et al* 2007; Rowan *et al* 2008; Niromanesh *et al* 2012) and one study was blocked randomization with un-blinded trial (Ijas *et al* 2010). Regarding reproducibility of studies, information on insulin intervention was very limited and adequate information were only available in only 5 studies (Moore *et al* 2007; Niromanesh *et al* 2012; Spaulonci *et al* 2013; Mesdaghinia *et al* 2013; Ruholamin *et al* 2014). Furthermore, 3 studies did not report explicitly on operational definition of some outcomes being reported (e.g. neonatal hypoglycemia) (Moore *et al* 2007; Spaulonci *et al* 2013; Mesdaghinia *et al* 2013).

Table 2.5.Quality assessment of NRCTs studies (Metformin vs Insulin)

Study ID	Internal Validity										External Validity				Potential bias
	Selection			Performance		Detection	Attrition		Reporting	Others					
	Consecutive sample	Unbiased Selection Criteria	Baseline Comparability	Same care pathway	Favours group	Blinding of outcome assessors	Prior sample size	Intention-to-treat analysis	Complete outcome report	Confounders controlled	Population	Intervention	Comparator	Explicit reported outcomes	
Tertti2008	-	+	-	+	M	-	+	+	+	-	+	+	-	+	High
Balani 2009	+	-	-	+	M	-	-	-	-	-	-	+	-	+	High
Rai 2009	+	?	?	-	M	-	-	+	-	-	+	+	-	+	High
Goh 2011	-	-	-	+	M	-	-	+	-	-	+	-	-	+	High
Gandhi 2012	-	-	+	+	+	-	-	+	-	-	-	-	-	-	High
Iftakhar 2012	+	?	-	-	M	-	-	+	-	-	+	+	-	+	High

('+' means appropriate, '-' means not done or not mentioned or not appropriate or '?' means inadequate data to justify that the study was done appropriately. For baseline comparability and favours group, there were 2 separate items being consider:1) whether the maternal baseline which were considered important in GDM(Age, BMI, parity, glucose levels at OGTT, ethnicity, smoking, previous history of still-birth were considered and reported and 2) if they were unequal across arms, which group could be potentially advantaged out of this biases? D= Diet, I= Insulin, M=metformin, G=glibenclamide, MF= Metformin Failure

2.3.1.1.2 Observational Studies (Table 2.5)

Three out of six studies were recruited all eligible GDM in defined setting and time frame. However, one study excluded metformin failure group from metformin arm; one study had compulsory insulin criteria; one study had mixed population with di*et al*one group in non-metformin arm. Moreover, Rai 2009 study included type 2 diabetes (<20%) but the percentage was not comparable across 2 groups. Expectedly, only one study had fulfilled to have comparable maternal baseline characters between metformin and insulin groups whereas other studies have less insulin resistant group in metformin arm. Moreover, only Tertti 2008 study performed power calculation. Yet, 5 out of 6 studies analysed all women in either group as they were initially allocated. The pregnancy outcomes reported were found to be complete in only one study to detect birth weight and incidence of hypoglycemia. Although some studies considered to have controlled on some of the confounders (e.g. studies done by Tertti *et al* and Balani *et al*, all eligible women for metformin in the study period were included with only case-matched number of insulin-treated participants (stratified by BMI and age), and equal number of participants, respectively), any of 6 NRCTs did adequate confounder controlled for any of their outcome measures. For extrapolation of the findings, the information on any of 6 studies were not adequate.

2.3.1.2 Description of characteristics of studies compared to insulin

Total of 15 studies with 18 relevant articles (2 studies with 3 extended offspring follow-up publications) were identified according to the selection criteria used in this review. Of these studies compared to insulin, 9 were RCTs and the rest 6 were NRCTs. A brief description on population involved and intervention strategy were given in table 2.6 and 2.7. Relevant abstracts are described in Appendix 2.3.

Population: There are a total of 15 studies, studying pregnancy outcomes of metformin in GDM compared to insulin (9 RCTs and 6 NRCTs). Out of 9 RCTs, 6 RCTs used 75 OGTT test to identify GDM whereas 5 out of 6 NRCTs applied (Table 2.3) whereas the other studies used 100 g OGTT to diagnose GDM. Two groups had conducted both RCTs and NRCTs, one each, with similar intervention strategy (Goh *et al* 2011; Tertti *et al* 2013; Rowan *et al* 2008; Tertti *et al* 2008). All the studies included all GDM diagnosed by OGTT tests (i.e. both moderate and severe hyperglycemic GDM), except Tertti 2013 study where GDM diagnosed above FPG >7 mmol/L and 2hr PP>11mmol/L excluded from randomization. Moreover, there was compulsory insulin to GDM with FPG >7 mmol/L and PP >10mmol/L in Tertti 2008 NRCTs study. It should also be noted that 13% (8/60) women included in Rai 2009 study were type 2 diabetes, with 2/30(6.7%) in metformin arm and 6/30(20%) in insulin arm. Moreover, GDM women included in both arms were conducted in the same setting except Tertti 2008, Iftakhar 2012 study and Rai 2009 study.

Intervention: The maximum dose of metformin varied from 1500 to 3000 mg. In all RCTs and majority of NRCT studies, insulin was given to metformin-treated GDM only after maximum dose of metformin failed to meet the target glycemic levels.

However, in one NRCTs, e.g. Goh 2011, insulin was given to GDM with FBS >6mmol/L at initiation of metformin. In all studies, all GDM who received metformin (i.e metformin alone or metformin plus supplementary insulin) were included in metformin arm whereas all GDM included in non-metformin/insulin arm were diet-failed GDM with insulin therapy. Yet, in some studies, e.g Balani 2009, Mesdaghinia 2013 and Ruhoalmin 2014, GDM with metformin therapy alone were included in metformin arm,but some of the outcomes of metformin plus insulin group in Balani 2009 study were separately reported and presented in combination in our meta-analysis. Moreover, metformin was started as 500mg od/bd and titrated against blood glucose levels over a week or two in almost all studies, except Ijas 2010 study where metformin was given as rather fixed dose administration for 3 weeks. Furthermore, Ijas 2010 and Tertti 2013 studies described that extended-released metformin was used. In addition, Tertti 2013 was the only study out of 15 studies included where vitamin B12 plus vitamin supplements given to metformin arm.

Comparator - The types of insulin used in insulin arm were a combination of long/intermediate acting insulin with regular insulin, but most of the studies did not report doses of insulin used. Among 5 studies with the dose and strategy reported, Tertti 2013 study used rather ‘targeted approach’method, i.e, if long/intermediate acting insulin was given to GDM women with higher fasting whereas short acting insulin was given to those with higher postprandial glucose. It should be noted that Ijas 2010 RCT, the dose of insulin requirement was higher in metformin group than insulin group (mean dose: 43 IU vs 30 IU, p=0.05). In this study, as described earlier, there was 3-weeks of titration period for metformin group before insulin was given. On the contrary, insulin dose requirement was higher in insulin group in the NRCT

studies of Iftakhar 2012 (mean±SD: 42.9±32.7 vs 60.8±39.9 units) and Gandhi 2012 (34.3±43.2 vs 41.3±32.8 units) whereas Tertti 2008 NRCT found no difference (median dose: 11 vs 10 units).

Outcomes: The primary outcomes considered varied from study to study(Table 2.6 and 2.7). The most frequent primary outcomes were neonatal birthweight and maternal glycaemic control.

Table 2.6. Description of RCTs studies (Metformin vs Insulin)

Study ID (study design)	Setting	Sample size	Participants' characters	Metformin	Insulin	Primary outcomes
Moore 2007 (RCT)	United states, University hospital of Mississippi Medical Centre	63	24-30 weeks gestation, screened by 50g OGCT 1hr≥140mg/dl,	500 mg bd, increased as necessary(maximum 1000mg bd).Insulin was started if metformin was failed(maximum dose with 2 glucose values exceeded target for 2 consecutive weeks); monitor 3 times daily	0.7units/kg of actual BWt(2/3 morning & 1/3 evening); a combination of regular insulin and NPH insulin; monitored 3 times daily	FBS, 2hrPP, MOD, incidence of shoulder dystocia, incidence of PPH
Rowan 2008 (RCT)	10 New Zealand & Australian urban obstetrical hospital	733	18- 45 years, singletons, 20-33 weeks GA, met hospital usual criteria for insulin treatment, more specific criteria avoided	(Metomin, Diaformin) started at 500 mg od/bd with food, increased over 1-2 weeks to meet glycemic targets up to maximum of 2500mg/d; stopped if maternal contraindications(liver or renal impair or sepsis) or FGR developed.Insulin added if target was not achieved with metformin.	Insulin prescribed according to ususal practice, typically SA insulin analog before meals and intermediate insulin once or twice daily	Composite outcome(neonatal hypoglycaemia, respiratory distress, birth trauma, APGAR < 7 and preterm)
Ijas 2010 (RCT)	Finland, Tertiary Oulu hospital & secondary Kainuu Central hospital	97	12-34 weeks GA, singletons, risk factor based screening(over 40 years old, BMI > 25 kg/m2, glycosuria, prior GDM, previous baby BWt> 4500 g, current suspected macrosomia)	Metformin(Diformin retard®) initiated at 750 mg od for 1st wk, bd for 2nd wk, tds from 3rd week onwards; stopped if significant side effects, such as diarrhoea developed. Supplemental insulin was added if normoglycemia was not achieved with maximum metformin daily dose in 1-2 wks	LA insulin(Protaphan®) for FBS and RA insulin(Humalog®) for PP control according to hospital guidelines	incidence of macrosomia, LGA using Finnish sex specific charts adjusted for gestational age
Hassan 2012 (RCT)	Pakistan, Lyari General Hospital, Dow University of Health Sciences and private maternity hospitals	150	18-45 years of age, singleton pregnancy, 20-36 weeks of gestation, those with high risk factors offered 50 g OCT, if ≥ 140 mg/dl --> 75 g OGTT	Metformin started at 500 mg, increased up to maximum of 3000 mg/day as patients' tolerance and glucose levels; stopped if significant preelampsia, sepsis, pregnancy cholestasis or IUGR developed or with drug intolerance due to GI SE.Insulin added if not control with metformin alone in 1-2 weeks; a combination of regular and intermediate human insulin before meals twice daily.	NR	macrosomia(>4000g)

Table 2.6. Description of RCTs studies (Metformin vs Insulin)

Study ID (study design)	Setting (Number)	Sample size	Participants' characters	Metformin	Insulin	Primary outcomes
Niromanesh 2012	Women Hospital, The Shariati Hospital and the Valiasr Hospital, University of Tehran, Iran	172	Screening by 50g OGCT;18-40 years old singletons between 20 and 34 weeks; excluded mothers with history of systemic underlying diseases(cardiovascular, renal, liver and autoimmune), substance abuse, overt diabetes mellitus(except previous GDM history) and major foetal malformation	Started at 500 mg bd; increased by 500-1000 mg ½ wks against target up to maximum of 2500 mg divided dose with meal, continued until delivery and added insulin if glycemic control not achieved with maximum metformin dose; 4 times/day monitor	Started with NPH 0.2units/kg for high FBS and short-acting insulin for high PP(1 unit for every 10 mg/dl over target) and if both high, total insulin dose of 0.7 units/kg(2/3 is NPH: 2/3 before breakfast and 1/3 before bedtime) and 1/3 is regular insulin:2/3 times), titrated against daily glucose monitoring; 4 times/day monitor	Maternal glycemic control and birth weight
Spaulonci 2013	Obstetrics Clinic Hospital, Sao Paulo, Brazil	92	Singletons, absence of risk factors for lactic acidosis(renal failure, chronic liver disease, severe chronic pulmonary disease, coronary insufficiency, history of thromboembolic phenomena) and absence of anatomic and/or chromosome anomalies of the conceptus detected by ultrasonography	Started at 850 mg tds and if not controlled, 850 mg qid; if still not controlled, added insulin; monitored 4 times/day	Started with NPH of 0.4 units/kg /day with half given before breakfast, ¼ before lunch and ¼ at 22 hours; monitor 7 times per day; adjusted weekly against glucose levels; if only postprandial is high, regular insulin added ½ hr before meal in addition.	Glycaemic control
Tertti 2013	Turku University Central Hospital, Finland	217	GDM diagnosed according to current criteria and selected if failed to diet therapy, and normal renal, liver and cardiac function and no metformin use 3 months prior and during this pregnancy	Started at metformin (Diformin retard®) 500 mg od for 2 days, 500 mg bd for 1 week, titrated up to maximum of 1 g bd; monitored 4 times daily 3 days a week; vitamin B compound containing 0.4 mg folic acid initiated along with metformin.	Initiated using NPH insulin(Protaphane®, Novo Nordisk, Bagsvaerd, Denmark) and/or rapid-acting insulin lispro (Humalog®, Lilly, Indianapolis, IN, USA) or insulin aspart (Novorapid®, Novo Nordisk)	Birthweight

Table 2.6. Description of RCTs studies (Metformin vs Insulin)

Study ID (study design)	Setting	Sample size	Participants' characters	Metformin	Insulin	Primary outcomes
Mesdaghinia 2013	Shabih Khani Hospital, Isfahan, Iran	200	18-45 years of age, single pregnancy, without history of diabetes prior to pregnancy with GA of 24-34 weeks; GDM screened by GCT and diagnosed by OGTT;poor glycemic control on lifestyle modification for a week	500 mg initially and adjusted up to 2500 mg/day; metformin-treated GDM who needed insulin were excluded from the study and replaced with new patients.	0.5IU/kg/day (2/3 NPH insulin and 1/3 regualar). And 2/3 given in the morning and 1/3 in the afternoon.; 1IU of NPH or regular insulin added appropriately for every 10mg/dl increase in blood glucose	Unclear
Ruholamin2014	Alzahra and Shahid beheshti hospital, Iran	100	18	500mg bd and adjusted up to 1500 mg/day; stopped if maternal contraindications(such as liver/renal impairment or sepsis), PIH or PE were developed. Metformin-treated GDM who required insulin were excluded from the study(n=2).	0.2IU/kg/day insulin, titrated to meet glycemic targets	Pregnancy-induced hypertension

Table 2.7. Description of NRCTs studies (Metformin vs Insulin)

Study ID	Setting (Number)	Sample size	Participants' characters	Metformin	Insulin	Primary outcomes
Tertti 2008 (retrospective cohort)	Finland, Turku University central hospital	90	11- 32 weeks GA, singletons, screening - BMI ≥25kg/m2, ≥40 years, previous macrosomia, glycosuria during pregnancy, previous GDM, suspected foetal macrosomia in current pregnancy,	Metformin started at 500 mg od to 750 mg bd, if FBS > 7 mmol/l and/or PP > 10 mmol/l, insulin was always started.	Intermediate acting insulin was usually started.	BWt, incidence of hypoglycemia
Rai 2009 (prospective cohort)	India, 2 different obstetric units	60 mothers /61 babies	Screening by 50g OGCT 1hr ≥ 140 mg/dl Also included type 2 diabetes (6.7% in metformin grop and 20% in insulin group)	Metformin - started at 500 mg tds, titrate to a maximum of 2000mg/day	Intermediate acting(Mixtard) and short acting insulin (Actrapid) used whenever required	Maternal glycemic control
Balani 2009 (Prospective cohort)	United Kingdom, University hospital	227	28 weeks GA	Metformin 500 mg bd with food initially, subsequently titrated up to maximum of 2500 mg daily, patients attended AN clinic after dose titration and remained in close contact with diabetes specialist team.Insulin added if control was not achieved depite maximum metformin dose. Included cases exclusively with metformin alone	Basal-bolus regimen of insulin aspart with meals and insulin glargine once daily(from case record data who had been managed by same diabetic and obstetric team according to same GDM care pathway)	Principal maternal outcome: weight gain from enrolment to delivery, preeclampsia and pregnancy-induced hypertension.
Goh 2011 (prospective cohort)	New Zealand, national's women health	864 mothers /877 babies	After 20 weeks GA, screening – 50 g OGCT 1hr plasma glucose ≥ 7.8 mmol/l	Dose on metformin was not reported. if FPG ≥ 6 mmol/l, bedtime isophane insulin was supplemented at baseline. SA insulin analogue was prescribed for metformin failure - maximum daily dose of metformin 2500 mg (3000 mg for morbidly obese), over 1-2 weeks failed to achieve targets.	bedtime Intermediate acting insulin analogue + premeal SA insulin analogue	NR

Table 2.7. Description of NRCTs studies (Metformin vs Insulin)

Study ID	Setting (Number)	Sample size	Participants' characters	Metformin	Insulin	Primary outcomes
Gandhi 2012 (retrospective cohort)	United Kingdom, Royal hallamshire hospital	592	NICE guidelines	Metformin initiated at diagnosis of GDM: 500 mg bd with food, increased to 1g bd, if tolerated, clinically appropriate with no side-effects, 1 week after; metformin was stopped if any side effects or obstetric complications(preeclampsia, cholestasis, IUGR)) was developed. glycemic control was considered to be abnormal if FBS > 6 mmol/l and/or 1hr PP > 8 mmol/l(145 mg/dl)	Non-metformin (subcutaneousinsulin±lifestyle advice)	NR
Iftakhar 2012 (Retrospective)	Retrospective historically controlled	93	< 34 weeks gestation at initiation of treatment; Insulin cohort(2006-07) and metformin cohort(2010); higher 2hrPP and increased proportion of previous GDM history in insulin group	Maximum of 2000 mg/d; median- 7 week duration, range 500-2000 mg	Isophane insulin at bedtime, short-acting insulin before meal	Weight gain and insulin requirement

GA- gestational age, AC – abdominal circumference, BWt – Birth weight, BP – blood pressure, GI SE – GI sideeffects, NR – Not reported, OGCT – Oral Glucose Challenge test, OG – obstetricians and gynaecologists, USG – Ultrasound, LFT – Liver function tests, FGR – Foetal growth restriction, AN clinic – antenatal clinic, SA insulin – short-acting insulin,RA insulin – rapid acting insulin, F-up – Follow up, USG – ultrasound,

2.3.1.3 Pregnancy outcomes (Metformin vs Insulin)

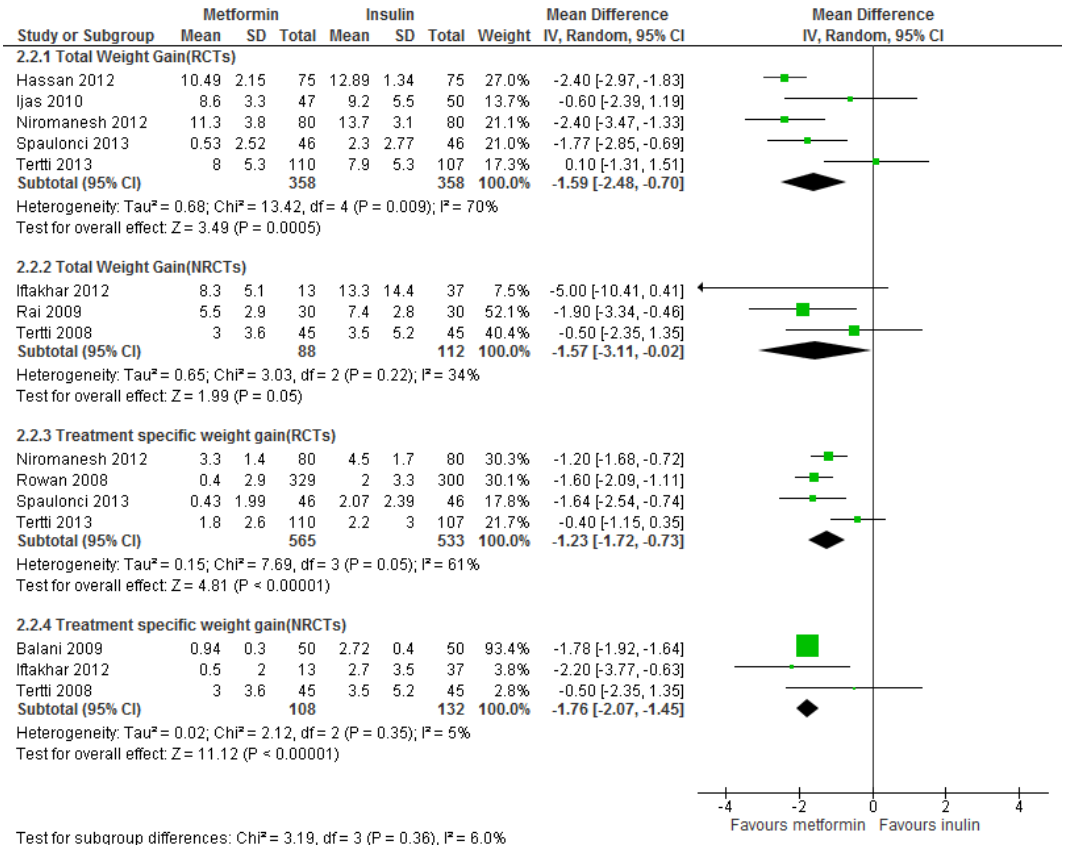
Maternal Outcomes

1. Maternal weight gain

There was reduced maternal weight gain with metformin treatment. The pooled estimates of 5 RCTs revealed Weighted Mean Difference (WMD) of -1.59 (-2.48, -0.70)kg and that of 3 NRCTs was WMD of -1.57(-3.11, -0.02) kg. Moreover, there was significantly lower weight gain was observed when the weight gain was limited to during medication period, WMD of 4RCTs -1.23(-1.72, -0.73) and that of 2NRCTs -1.78z9-1.92, -1.65). (Figure 2.2)

Sensitivity analysis: The statistical heterogenetiy (I^2) was 70% in random-effect RCT meta-analyses) where the effect estimates of Ijas 2010 and Tertti 2013 were found to be much different. These two studies were reported to use extended-release metformin in compared to other RCTs. When these two studies were excluded, the summary effect estimate changed to WMD of -2.29(95%CI: -2.75, -1.83) kg with I^2 of 0%. In NRCTs metaanalyses, I^2 was 34% which was much attributable to Tertti 2008 study. Although the study did not mention about the type of metformin use, considering being conducted by the same group of Tertti 2013 study and having comparable outcome to them, it was likely that they might use extended release metformin and thus excluded for sensitivity analysis. After exclusion, the pooled effect estimate of total weight gain was found to be WMD of -2.31 (95%CI : -4.37, -0.25) with I^2 of 0%.

Figure 2.2. Forest-plots of Maternal Weight Gain (kg)



2. Glycaemic control

Average Fasting glycaemic control : Four RCTs (n=1048) and one NRCT (n=60) reported the effects of metformin on fasting plasma glucose (FPG) control (Fig 2.3 A). There was no clear difference in maternal fasting glucose levels between the groups in the RCTs (WMD 0.34[95%CI -1.74, 2.42]) mg/dL. Three of them assessed glycemic control as a primary outcome measure (Moore *et al* 2007; Niromanesh *et al* 2012; Spaulonci *et al* 2013).

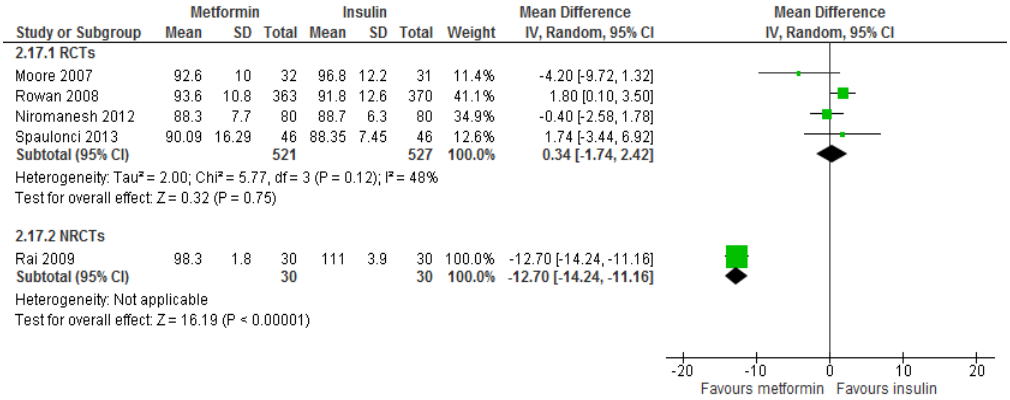
Average Postprandial glycaemic control: Metformin use could be possibly associated with a lower postprandial maternal glucose in 4 RCTs (n=1048), (WMD -2.29 mg/dl [95% CI -4.65, 0.07]) The issue with the NRCT has been discussed above. The largest RCT found a significantly lowered postprandial glucose with metformin treatment [178](Fig 2.3 B).

Sensitivity analysis: There was large (>40%) statistical heterogeneity in RCTs meta-analyses of FPG and PP results. It might be due to differences in insulin dose administration in insulin arm (i.e. start dose and type of insulin use). The largest Rowan 2008 RCT did not describe any information on details of insulin use. Moreover, as described in description of intervention, in Niromanesh 2012 study, target approach of insulin was used.

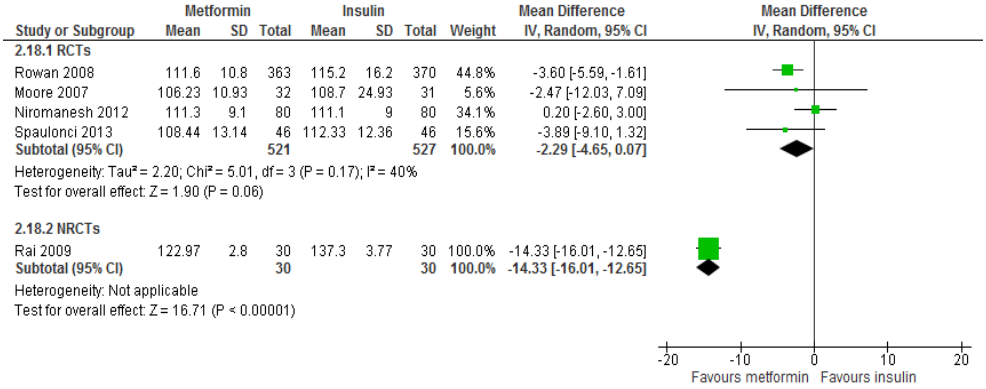
Glycaemic control 1 week after randomization: 2RCTs have reported on glycemic levels of 1 week after randomization. It was found that GDM women with metformin had lower 2hr post-prandial glycemic levels in 1 week after randomization[WMD -3.25(95%CI: -5.34, -1.16)mg/dL] although there was no observed difference in fasting glucose levels [WMD 0.52 (95%CI: -2.12, 3.16) mg/dL] .

Figure 2.3 Forest-plots of glycaemic control

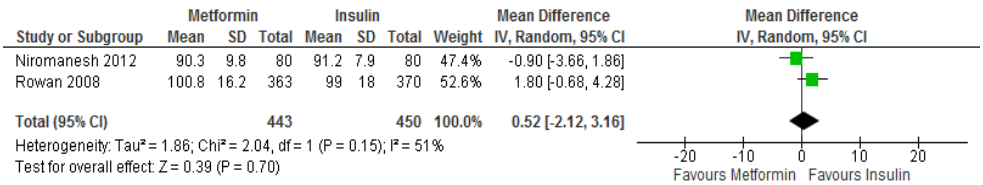
A) Average Fasting Plasma Glucose Control (mg/dL)



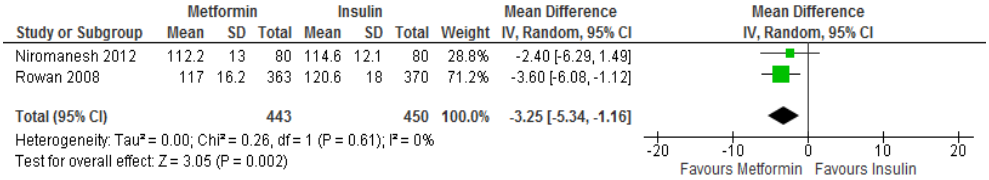
B) Average Postprandial Glucose Control (mg/dL)



C) Fasting Plasma Glucose Control 1-week after randomization (mg/dL)



D) Postprandial Glucose Control 1-week after randomization (mg/dL)



3. Hypertenison in Pregnancy

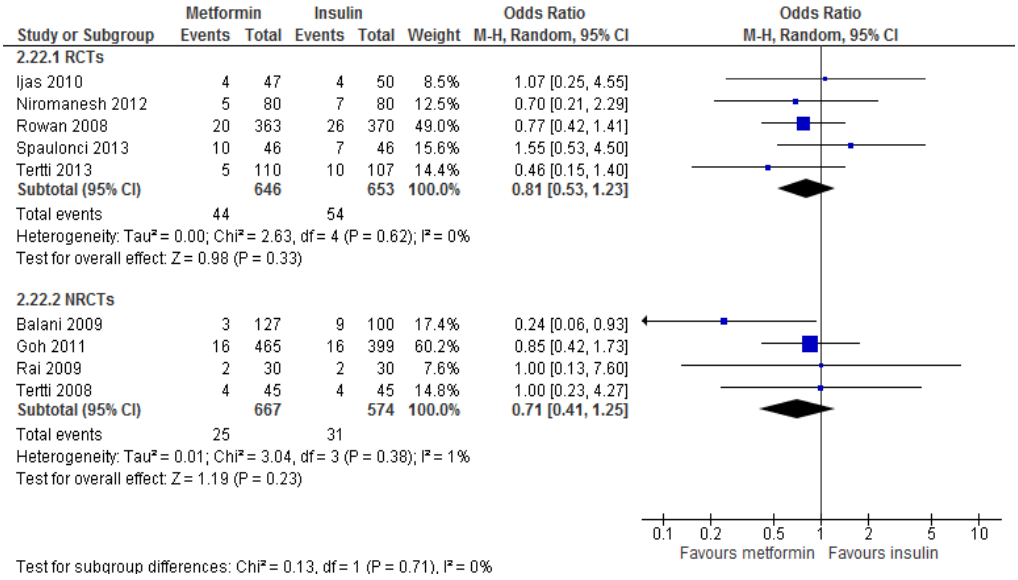
The reports on outcomes of hypertension in pregnancy varied: some studies reported pregnancy-induced hypertension (PIH) only or pre-eclampsia only or both.

The incidence of preeclampsia in association with metformin use was examined in 5 RCTs (n=1082) and 4 NRCTs (n=1241) (Fig 2.4). There was no difference in the incidence of preeclampsia between the two treatment arms (OR 0.81 [95%CI 0.53,1.23]) and (OR 0.71 [95% CI 0.41, 1.25]) for RCTs and NRCTs respectively (Figure 2.4 A).

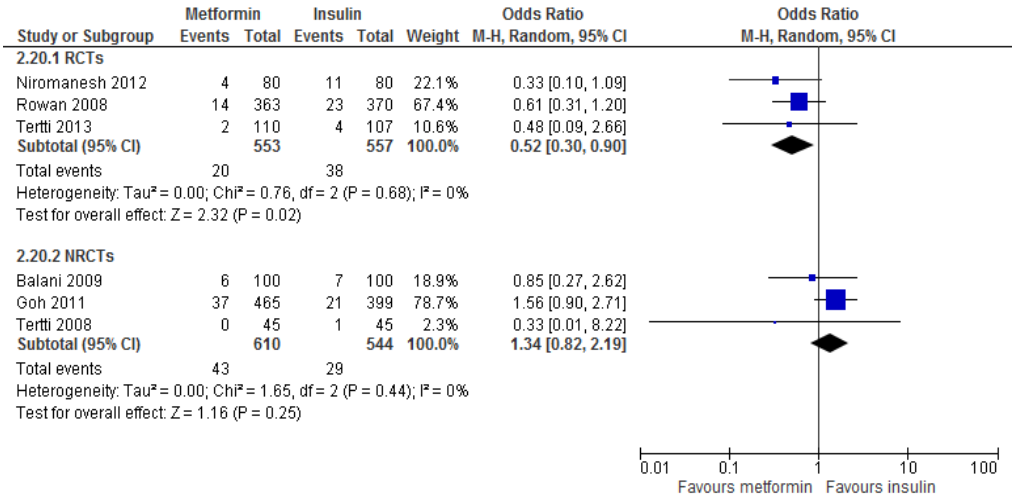
However, there was significant difference in metaanalyses of PIH with 2 RCTs (OR: 0.52 [95%CI : 0.29 vs 0.94]) with reduction in metformin arm. (Figure 2.4 B)

Figure 2.4. Forest-plots of hypertension in Pregnancy

A) Pre-eclampsia



B) Pregnancy-induced hypertension (PIH)



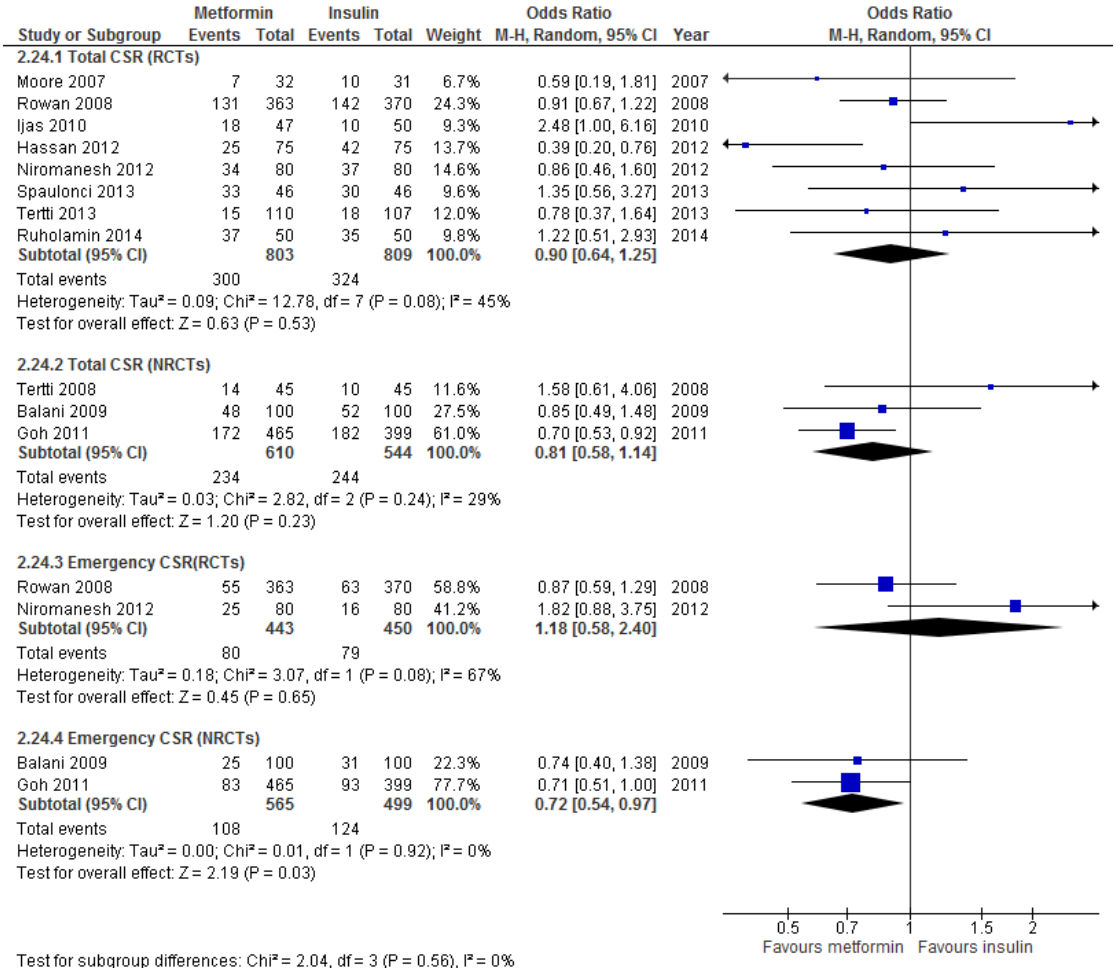
5. Caesarean section rates

Eight RCTs (n=1612) and 3 NRCTs (n=1154) were examined to assess the effects of metformin treatment on caesarean section rates (Figure 2.5). Caesarean section rate was found to be high in treated GDM in all included studies. Up to half of them delivered by caesarean section. Caesarean section rates were not a primary outcome measure in any of the studies and only 2 studies had reported emergency caesarean section rates (CSR) separately. Among total CSR, there was no difference in caesarean section rates between the two treatment arms in either the RCTs group (odds ratio 0.90 [95% CI 0.64, 1.25]) or the NRCTs group (odds ratio 0.81 [95% CI 0.58, 1.14]). Among emergency CSR, although there was similar CS rates in RCTs meta-analysis, the NRCTs meta-analysis showed that there was reduced CS rates with metformin treatment with OR of 0.72(95% CI 0.54, 0.97).

Sensitivity analysis: There was high heterogeneity in RCTs meta-analysis of total CSR (I^2=45%) which was mainly contributed by Ijas 2010 study and Hassan 2012 study. It should be noted that fixed dose metformin administration was offered in Ijas 2010 study over 3 weeks whereas the highest maximum dose of metformin dose 3000mg was used in Hassan 2012 study. After exclusion, the pooled effect was similar with reduced confidence interval [OR 0.91(95%CI 0.73, 1.15)]. In NRCTs meta-analysis of total CSR, the heterogeneity was attributable to Tertti 2008 study. In this study, metformin was not offered to GDM women with FPG >7 mmol/L and PP >10mmol/L. Interestingly, despite presumably higher proportion of much higher glycemic levels in insulin arm, the CS rate was lower. In contrast, as mentioned before, Goh 2011 study had compulsory insulin to certain GDM group with metformin initiation and it was found that the CS rates was significantly reduced in metformin arm. This might

indicate that if metformin is offered to GDM women with much higher FPG levels, earlier insulin initiation might have favourable CS rates.

Figure 2.5. Forest-plot of Caesarean Section Rates



6. Induction of labour

No difference in the rate of induction of labour was detected, in either RCTs meta-analysis (OR 0.89 (95% CI 0.61, 1.28) and NRCTs meta-analysis (OR 1.24 (95% CI 0.84, 1.82), reported by 5 RCTs and 3 NRCTs .

Sensitivity analysis: The heterogeneity of I^2=38% in RCTs meta-analysis was attributable to Tertti 2013 study. This group had done one RCT and one NRCT (Tertti 2008) with similar GDM management strategy and reported much different results. The Tertti 2013 study was different from other included studies in that it excluded GDM women with FBS > 7mmol/L or 1hr PP > 11 mmol/L from randomization. This means that the population included only GDM women with moderate hyperglycaemia. Thus, metformin effect might be exaggerated in this study. After exclusion of this study, the effect changed to OR of 0.98 (95%CI 0.76, 1.27) with nil heterogeneity.

We are unable to synthesise the data for the other maternal outcomes due to non-reporting or relative heterogeneity in their outcome definition.

Table 2.8. Important maternal outcomes of GDM (Metformin vs Insulin) stratified by 75g/100g OGTT

Outcomes	Types of studies	Sample size	WMD/RR	I^2	Subgroup analysis (n)	WMD/RR	I^2
1. Maternal weight gain					75g (4)	-1.32(-2.48,-0.17)	76%
i) Total weight gain	RCTs	716	-1.59(-2.48,-0.70)	70%	100g(1)	-2.40(-3.47,-1.33)	
	NRCTs	200	-1.57(-3.11,-0.02)	34%	75g(2)	-2.00(-6.16,2.16)	58%
					100g(1)	-1.90(-3.34,-0.46)	
ii) Specific weight gain	RCTs	1098	-1.23(-1.72,-0.73)	61%	75g(3)	-1.22(-2.01,-0.44)	74%
					100g(1)	-1.20(-1.68,-0.72)	
	NRCTs	150	-1.78(-1.92,-1.65)	0%	75g(2)	-1.76(-2.07,-1.45)	5%
2. Glycaemic control					75g(2)	1.79(0.18,3.41)	0%
i) FPG	RCTs	1048	0.34(-1.74,2.42)	48%	100g(2)	-1.42(-4.72,1.88)	37%
	NRCT	60	-12.70(-14.24, -11.16)		100g(1)	-12.70(-14.24,-11.16)	
ii) PP	RCTs	1048	-2.29(-4.65,0.07)	40%	75g(2)	-3.64(-5.50,-1.78)	0%
					100g(2)	-0.01(-2.70, 2.68)	0%
	NRCT	60	-14.33(-16.01, -12.65)		75g(1)	-14.33(-16.01,-12.65)	
iii) HbA1c	RCTs	1294	-0.00(-0.15,0.15)	86%	75g(3)	0.06(-0.15,0.28)	91%
					100g(2)	-0.12(-0.37,0.14)	75%
3. Pregnancy induced hypertension(PIH)	RCTs	893	0.52(0.29,0.94)	0%	75g(2)	0.59(0.31,1.10)	0%
					100g(1)	0.33(0.10,1.09)	
	NRCTs	1154	1.34(0.82,2.19)	0%	75g(3)	1.34(0.82,2.19)	0%

Table 2.8. Important maternal outcomes of GDM (Metformin vs Insulin) stratified by 75g/100g OGTT

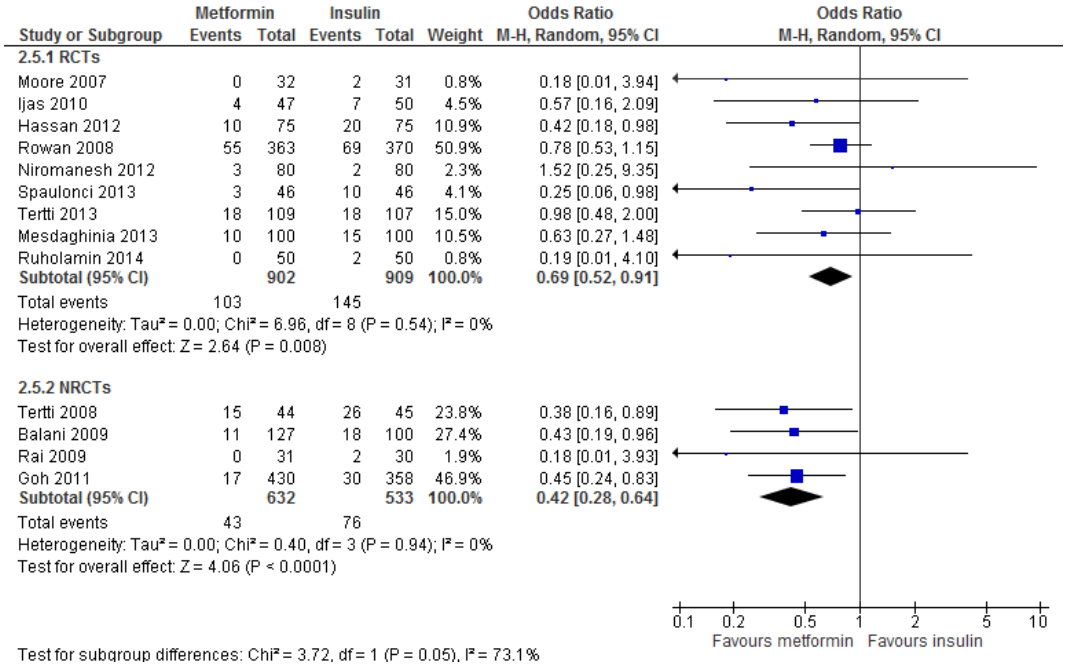
Outcomes	Types of studies	Sample size	WMD/RR	I^2	Subgroup analysis (n)	WMD/RR	I^2
4. Pre-eclampsia	RCTs	1299	0.81(0.53,1.23)	0%	75g(4)	0.83(0.53,1.30)	0%
					100g(1)	0.70(0.21,2.29)	
	NRCTs	1241	0.71(0.41,1.25)	1%	75g(3)	0.65(0.30,1.40)	32%
					100g(1)	1.0(0.13,7.60)	
5. Caesarean section rate					75g(6)	0.96(0.62,1.47)	59%
i) Total	RCTs	1612	0.90(0.60,1.25)	45%	100g(2)	0.76(0.46,1.36)	0%
	NRCTs	1154	0.81(0.58,1.14)	29%	75g(3)	0.81(0.58,1.14)	29%
ii) Emergency	RCTs	893	1.18(0.58,2.40)	67%	75g(1)	0.87(0.59,1.29)	
					100g(1)	1.82(0.88,3.75)	
	NRCTs	1064	0.72(0.54,0.97)	0%	75g(2)	0.72(0.54,0.97)	0%
6. Induction of labour	RCTs		0.89(0.61,1.28)	38%	75g(4)	0.88(0.59,1.29)	49%
					100g(1)	3.04(0.12,75.69)	
	NRCTs		1.24(0.84,1.82)	0%	75g(3)	1.24(0.84,1.82)	0%

Neonatal outcomes

1. Neonatal Hypoglycaemia

Among 8 studies, 6 RCTs (n=1295) and 4 NRCTs (n=1165) reported the outcome. Whilst the definition of neonatal hypoglycaemia varied from less than 1.6 to 2.6 mmol/l or the need to inject IV dextrose, all these studies,except Niromanesh 2012 study, reported decreased risk of neonatal hypoglycaemia with metformin treatment. Yet, there was no statistical heterogeneity in summary outcome estimate. It should be noted that Niromanesh 2012 did not mention to collect the outcome in their method section though they reported the outcome in the results. Random effect meta-analysis of both RCTs and NRCTs revealed that the rate of neonatal hypoglycaemia was significantly lower in infants born to metformin treated mothers than those to insulin-treated mothers, (OR 0.69 [95% CI 0.52, 0.91) in RCT and (OR 0.42 [95% CI 0.27, 0.64) for the NRCT group (Figure 2.6).

Figure 2.6. Forest-plot of Neonatal Hypoglycaemia



2. Large for gestational age (LGA)

Definition of LGA was similar across the included studies, with birth weight greater than 90th centile except the Ijas study which used birth weight of >2 standard deviation (SD) of the mean.

6 RCTs (n=1502) and 3 NRCTS (n=1696) reported the outcomes (Figure 2.7. A). Only the Ijas study examined LGA as a primary outcome measure. The RCTs did not show any difference in the incidence of LGA (OR 0.77 [95% CI 0.55, 1.08]) whilst the NRCTs showed a decreased incidence of LGA in the metformin treated group (OR 0.60 [95%0.46, 0.77]). It should be noted that Niromanesh 2012 and Spaulonci 2013 studies did not mention including LGA as outcome variable in their method section.

3. Small for gestational age (SGA)

Data on SGA were available from 7 RCTs (n=1548) and 4 NRCTs (n=1255) (Figure 2.7. B). There was no difference in pooled estimate risk of SGA in RCT meta-analyses (OR 0.98 [95%CI: 0.64, 1.50]) between metformin and insulin treatment. The data reported by NRCTs was found to be reduction in SGA risk with metformin than insulin (OR 0.66[95% CI 0.46, 0.96]). The variation between RCTs and NRCTs could be due to exclusion of metformin given to GDM women with possible intrauterine restricted babies in one NRCTs(Goh *et al* 2011) and differences in population characters favouring metformin arm (Rai *et al* 2009, Tertti *et al* 2008) which might paradoxically increase the number of SGA in insulin arm.

4. Macrosomia

Macrosomia was defined as > 4kg in 11 studies: 8 RCTs (n=1082) and 3 NRCTs (n=909) (Figure 2.7. C). The random-effect meta-analyses showed possible difference in the rate of macrosomia non-significantly (OR 0.71 [95%CI 0.46, 1.09]) in RCT meta-analysis and significantly (OR 0.63 [95%CI 0.42, 0.93]) in NRCTs meta-analysis. The Ijas and Hassan studies examined macrosomia as its primary outcome.

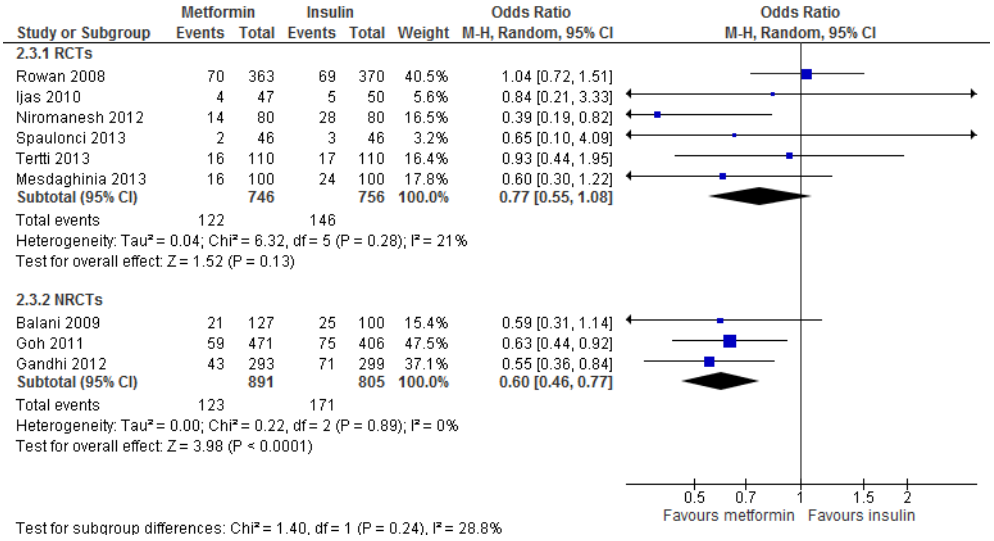
4. Foetal birthweight

Foetal birthweight was not significantly different between metformin and insulin treated groups in 9 RCTs (n=1815) (WMD -58.38 [95%CI -119.09, 2.32] grams and 4 NRCTs meta-analyses (n=1255) (WMD -35.37 [95%CI -134.56, 63.82] grams (Figure 2.7. D).

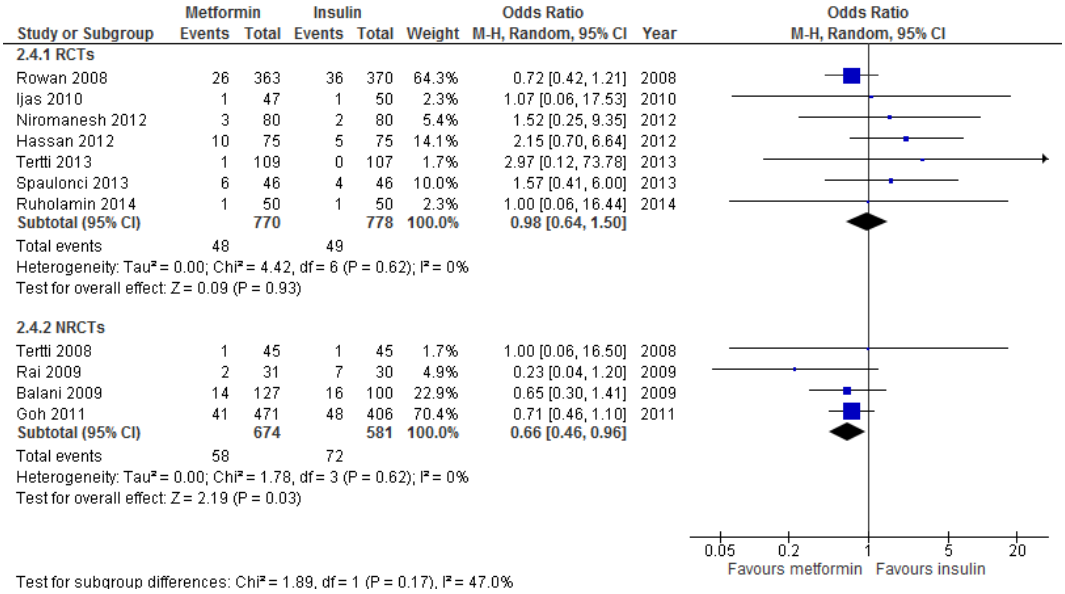
Sensitivity analysis: The statistical heterogeneity of I^2 of 33% was much influenced by Ijas 2010 and Hassan 2012 studies which results were in opposite direction. The methodology difference in these 2 studies were discussed in CS rate outcomes. After exclusion of these 2 studies, the effect changed to WMD of -50.04(95%CI -100.73, 0.64) grams with I^2 of 0%. Among NRCTs meta-anlaysis, I^2 of 43% was attributable to the result of Goh 2011 study. This was the study done by the same group as Rowan 2008 RCT study but the effects were found to be opposite. The strategy of metformin administration was different in Goh 2011 study in that GDM women in this study were not offered metformin if the foetal abdominal circumference was $<10^{th}$ percentile by ultrasound and thus, might possibly reduce birth weight in insulin arm. After exclusion, the effect of NRCTs meta-analysis changed to WMD of -98.59 (95%CI -199.89, 2.71) grams.

Figure 2.7. Forest-plots of Baby sizes at birth

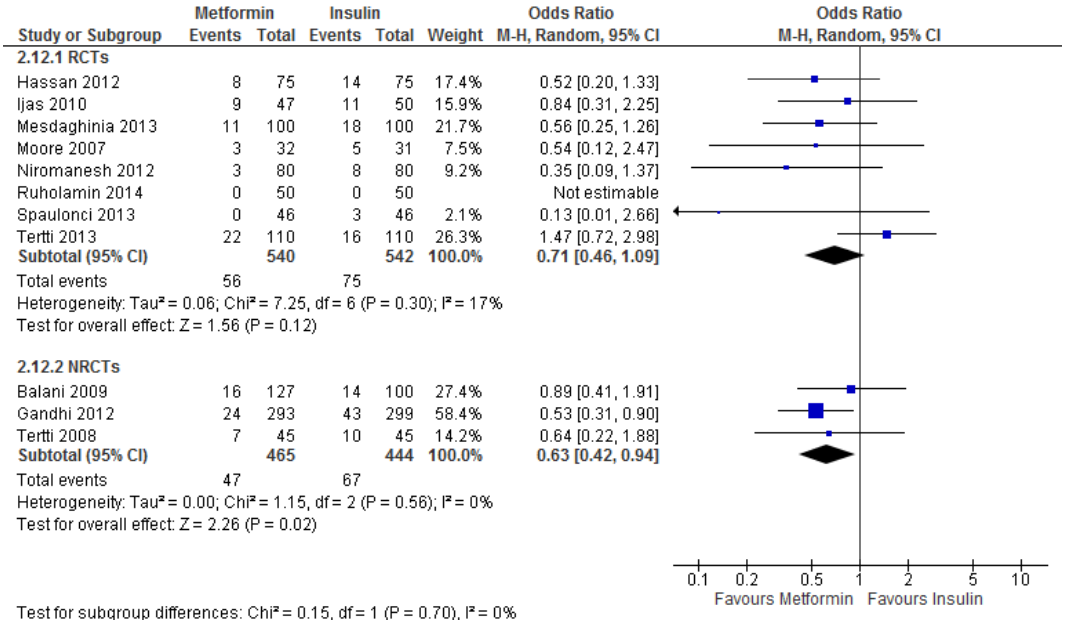
A) Large for Gestational Age



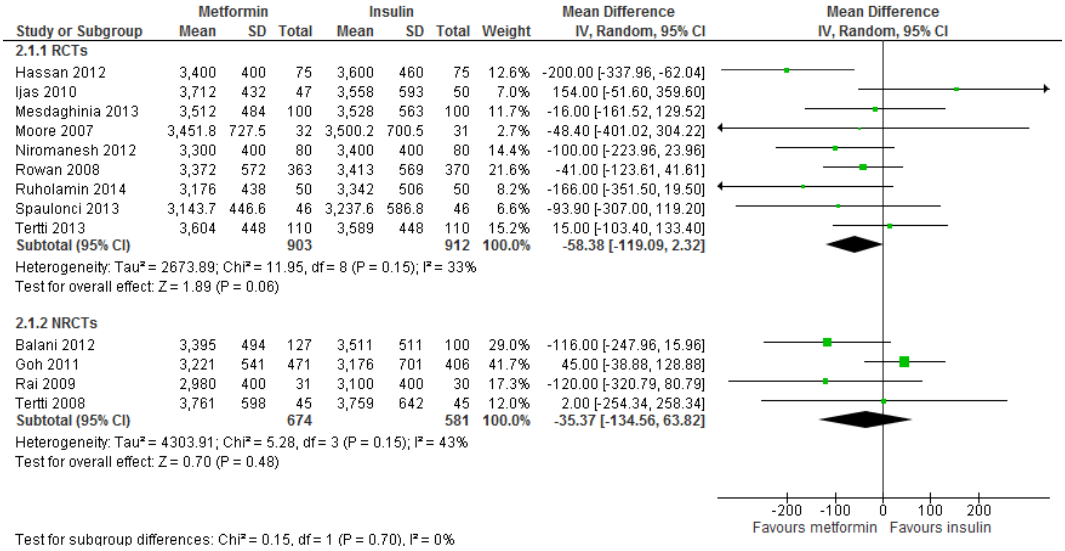
B) Small for Gestational Age



C) Macrosomia



D) Neonatal Birthweight (grams)



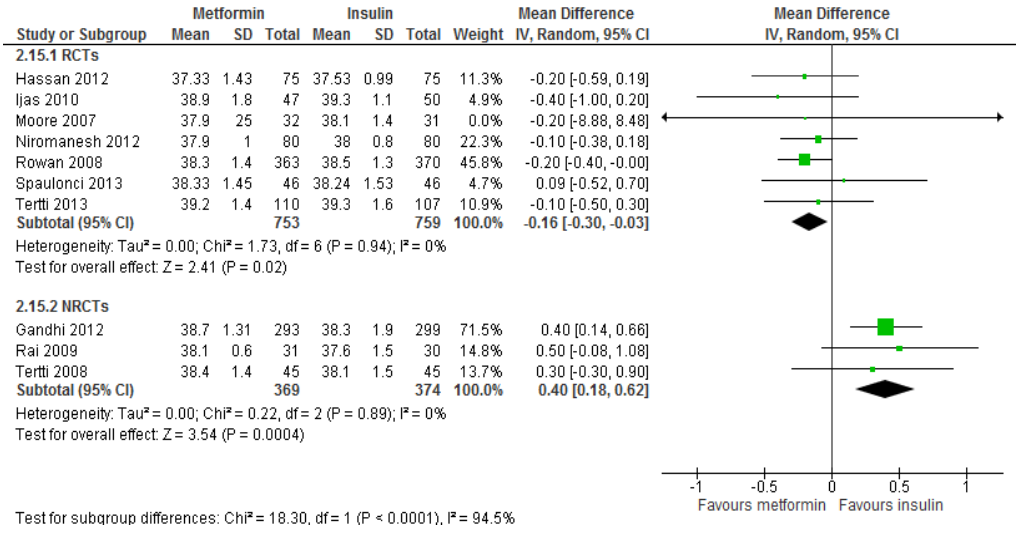
6. Gestational maturity

There was in the reduction of gestational age at delivery with metformin therapy in GDM in 7RCT meta-analysis (WMD -0.10(-0.29, -0.09) weeks whereas the summary estimate of 3NRCTs showed significant difference with much mature babies in insulin arm(WMD 0.4[95%CI 0.18, 0.62]) weeks (Figure 2.8. A). There was an increased risk of preterm delivery (< 37 weeks) with metformin treatment with OR of RCTs meta-analysis 1.22 (95% CI 0.63, 2.39) and that of NRCTs (OR 0.53 [95%CI 0.19, 1.49]) with high heterogeneity I^2=40% and 42% respectively. (Figure 2.8. B)

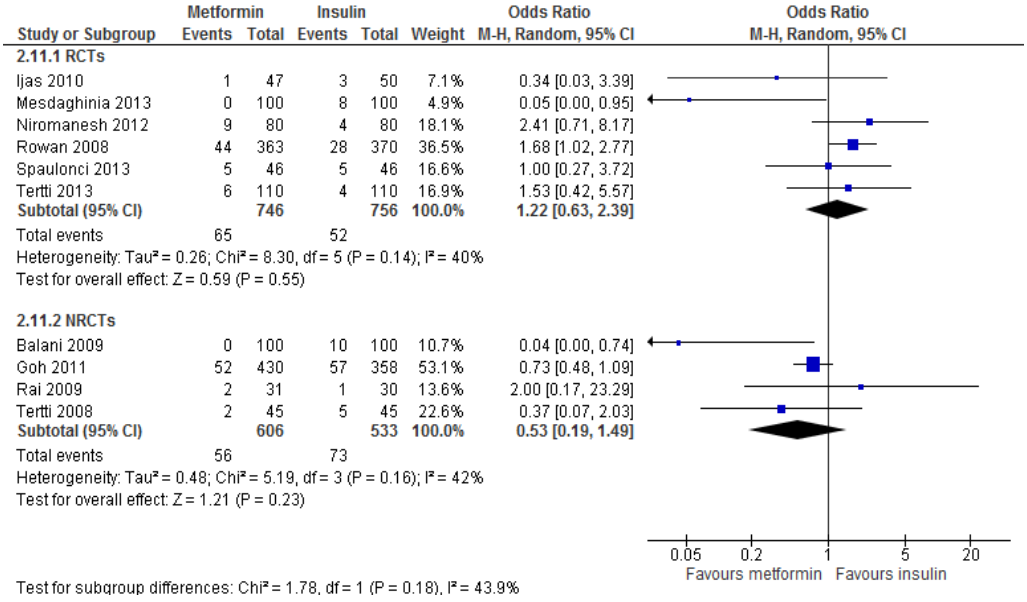
Sensitivity analysis: Among RCTs, the Mesdaghinia 2013 study excluded metformin-treated GDM with additional insulin group from metformin arm whereas all other studies included them. Thus, sensitivity analysis was performed with exclusion of this study and found that there was significant increased risk of prematurity with the odds of 1.57(95% CI 1.05, 2.36) with I^2=0% for RCTs meta-analysis. Similarly, among NTCTs, Balani 2009 study included metformin monotherapy group in their metformin arm whereas Goh 2011 study added supplementary insulin to GDM women with FBS ≥6mmol/L with metformin initiation. These two studies consistently reported that prematurity was increased in insulin-treated GDM compared to metformin-treated GDM with 19.2% vs 12.5% in Goh 2011 study and 10% vs 0% in Balani 2009 study. After exclusion of these 2 studies, the pooled estimated risk changed to OR of 0.68 (95%CI 0.14, 3.27).

Figure 2.8. Forest-plots of gestational maturity at delivery

A) Gestational age at delivery (weeks)



B) Prematurity (<37 weeks)



7. Neonatal jaundice

All the studies defined neonatal hyperbilirubinaemia or jaundice as the need for phototherapy apart from Moore *et al*, who defined jaundice as a bilirubin>5mg/dl and Rai *et al* who used >12mg/dl as a cut off(Moore *et al* 2007; Rai *et al* 2009). Analysis of the 9 RCTs (n=1811) revealed a possible reduction in the risk of neonatal jaundice with metformin use [OR 0.79; 95% CI 0.53, 1.18]. Analysis of the 3 NRCTs (n=378) showed similar non-significant reduction with metformin treatment [OR 0.51; 95% CI 0.19, 1.42] (Figure 2.9). There was large statistical heterogeneity in effect sizes which could be due to difference in characteristics of GDM population.

Sensitivity analysis: The heterogeneity in RCTs meta-analysis was much influenced by Niromanesh 2012 study which was the only study favouring insulin arm. The methodology of this study differed in that insulin was administered targeted approach compared to other study. Among NRCTs meta-analysis, because of much variations in studies conduct and paucity of number of NRCTs, the sensitivity was not performed.

Figure 2.9. Forest-plot of Neonatal Jaundice

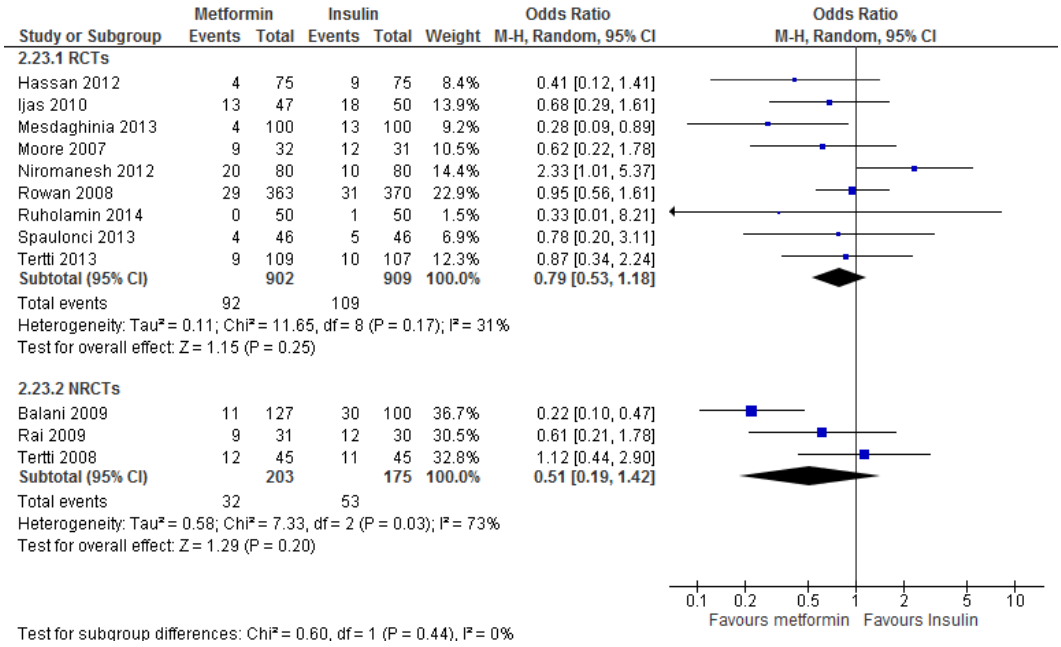


Table 2.9: Important Neonatal outcomes (Metformin vs Insulin) stratified by 75g/100g OGTT

Outcomes	Numbers of studies	Sample size	WMD/OR	I^2	Subgroup Analysis(n)	WMD/OR	I^2
1. Weight at birth					75g(6)	-59.96(-146.37,30.44)	55%
iii) Birth weight	9RCTs	1498	-58.38(-119.09,2.32)	33%	100g(3)	-63.59(-154.74,27.57)	0%
	4NRCTs	1255	-35.37(-134.56, 68.82)	43%	75g(3)	-18.71(-133.68,96.27)	51%
					100g(1)	-120.00(-320.79,80.79)	
iv) Macrosomia	8RCTs	1082	0.71(0.46,1.09)	17%	75g(5)	0.83(0.43,1.62)	37%
					100g(3)	0.50(0.27,0.95)	0%
	3NRCTs	909	0.63(0.42,0.94)	0%	75g(3)	0.63(0.42,0.94)	0%
v) LGA	6RCTs	1502	0.77(0.55,1.08)	21%	75g(4)	1.0(0.73,1.37)	0%
					100g(2)	0.49(0.30,0.82)	0%
	3NRCTs	1696	0.60(0.46,0.77)	0%	75g(3)	0.60(0.46,0.77)	0%
vi) SGA	7RCTs	1548	0.98(0.64, 1.50)	0%	75g(6)	0.96(0.62,1.48)	0%
					100g(1)	1.52(0.25,9.35)	
	4NRCTs	1255	0.66(0.45,0.95)	0%	75g(3)	0.70(0.48,1.02)	0%
					100g(1)	0.23(0.04,1.20)	
2. Neonatal hypoglycaemia	9RCTs	1811	0.69(0.52,0.91)	0%	75g(6)	0.67(0.48,0.94)	8%
					100g(3)	0.68(0.32,1.44)	0%
	4NRCTs	1165	0.42(0.27,0.64)	0%	75g(3)	0.43(0.28,0.65)	0%
					100g(1)	0.18(0.01,3.93)	0%
3. GA at birth	7RCTs	1512	-0.10(-0.29,0.09)	37%	75g(5)	-0.18(-0.33,-0.03)	0%
					100g(2)	-0.10(-0.38,0.18)	0%
	3NRCTs	743	0.40(0.18,0.62)	0%	75g(2)	0.38(0.14,0.62)	0%
					100g(1)	0.50(-0.08,1.08)	
4. Premature baby	6RCTs	1502	1.22(0.63,2.39)	40%	75g(4)	1.49(0.97,2.29)	0%
					100g(2)	0.44(0.01,24.48)	85%
	4NRCTs	1139	0.53(0.19,1.49)	42%	75g(3)	0.39(0.11,1.41)	55%
					100g(1)	2.0(0.17,23.29)	

Table 2.9: Important Neonatal outcomes (Metformin vs Insulin)stratified by 75g/100g OGTT

Outcomes	Numbers of studies	Sample size	WMD/OR	I2	Subgroup	Outcomes	Numbers of studies
5. Neonatal jaundice	9RCTs	1811	0.79(0.53,1.18)	31%	75g(6)	0.79(0.55,1.15)	0%
					100g(3)	0.78(0.22,2.75)	79%
	3NRCTs	378	0.51(0.19,1.42)	73%	75g(2)	0.49(0.10,2.39)	86%
					100g(1)	0.61(0.21,1.78)	
6. Foetal distress at birth					75g(6)	0.79(0.46,1.38)	0%
i) Respiratory distress	9RCTs	1811	0.78(0.50,1.22)	0%	100g(3)	1.07(0.27,4.31)	63%
	3NRCTs	1194	1.23(0.52,2.91)	0%	75g(3)	1.23(0.52,2.91)	0%
ii) Cord blood pH	6RCTs	857	0.00(-0.01,0.01)	0%	75g(5)	0.01(-0.01,0.02)	0%
					100g(1)	0.0(-0.02,0.02)	
	1NRCT	90	0.00(-0.04,0.04)		75g(1)	0.00(-0.04,0.04)	
7. NICU admission rate	8RCTs		0.69(0.50,0.95)	23%	75g(5)	0.79(0.60,1.04)	0%
					100g(3)	0.63(0.18,2.15)	60%
	5NRCTs		0.57(0.40,0.82)	28%	75g(4)	0.63(0.47,0.84)	0%
					100g(1)	0.19(0.05,0.75)	
8. APGAR at 5 minutes	6RCTs	778	0.03(-0.12,0.18)	12%	75g(2)	0.05(-0.15,0.26)	29%
					100g(4)	-0.06(-0.34,0.21)	0%
	1NRCT	90	-0.10(-0.52,0.32)		75g(1)	-0.10(-0.52,0.32)	
9. Shoulder dystocia	7RCTs	1570	0.60(0.29,1.25)	0%	75g(4)	0.48(0.20,1.23)	0%
					100g(3)	1.16(0.26,5.10)	10%
	2NRCTs	317	2.40(0.25,23.38)		75g(2)	2.40(0.25,23.38)	

8. NICU admission rate

Data on rates of NICU admission was found in 13 studies, 8 RCTs and 5 NRCTs. Analysis of the RCTs meta-analysis revealed reduction of NICU admission is association with metformin usage (OR 0.69 [95% CI 0.50, 0.95]). The NRCT analysis also showed a significant reduction with (OR 0.57 [95% CI 0.40, 0.82]).

Sensitivity analysis: Among RCTs, GDM women in Mesdaghinia 2013 study and Ruholamin 2014 study were included metformin monotherapy group in metformin arm and this might exaggerate the summary estimate. After exclusion of these 2 studies, the effect was no longer significant with OR of 0.82 (95%CI 0.62, 1.07). In NRCT analysis, there was imbalanced Type 2 diabetes population in Rai 2009 study with 6.7% and 20% in metformin and insulin arm respectively and it was reported that the average duration of hospital study also differed with 2.5 days and 6.5 days respectively. After exclusion, the I^2 changed from 28% to 0%, and effect was still significant.

9. Perinatal mortality

Because of small number of incidence of perinatal mortality rates in GDM, Generic Inverse Variance Model was applied to combine the effect estimate. Analysis of 6 RCTs and 3 NRCTs revealed no difference in perinatal mortality rates between either of the treatment arms in the RCTs (OR 1.00 [95%CI 0.10, 9.72]) or the NRCTs (OR 0.21 [95% CI 0.02, 1.92]) .

Other outcomes

8. Birth trauma

The number of birth trauma among treated GDM was found to be very low (0.03%). Data on birth trauma was specifically reported in 4 RCTs (n=1197) and 1 NRCT (n=90).

1 large RCT (Rowan *et al* 2008) reported birth trauma (mild if bruises or abrasions at birth but resolved before 6 weeks postpartum) in 16 out of 363 infants born by metformin-treated women with mild birth trauma and 17 out of 370 (15 mild and 2 severe) infants born to insulin-treated women, both numbers were considerably higher than data reported by 1 small RCT (Ijas *et al* 2010) where 2 out of 50 infants in insulin group (attributable to clavicular fractures due to shoulder dystocia) with no incidence of birth trauma in metformin group were observed. Another RCT did not find any case of birth trauma in both groups (n=150) (Hassan *et al* 2012) One retrospective cohort reported 2/45 in insulin and 1/45 in metformin group (1 clavicular fracture in each group and 1 Erb's palsy in insulin group) (Tertti *et al* 2008). One prospective cohort also observed an increased risk of birth trauma in the insulin-treated mothers although the exact number was not reported (Rai *et al* 2009), the interpretation of this result should be sceptical because of higher proportion of type 2 diabetes mothers.

9. Major or minor congenital abnormality

Ijas *et al* reported that there was one oesophageal atresia in the metformin arm whereas one baby with congenital ovarian cyst was observed in insulin group. One observational study reported specific congenital defects. In the metformin group, there is one case of a baby born with trisomy who subsequently died at 2 months (Tertti *et*

al 2009). Niromanesh group found one with ventricular septal defect, one with talipes equinovarus and one with moderate bilateral hydronephrosis in insulin group whreas they observed one with small ventricular septal defect, one with atrial defect, two with talipes equinovarus and one with unilateral cleft lip.

10. Tolerability of treatment

Discontinuation of treatment was reported as being low in 4 studies (the largest proportion <8%)(Rowan *et al* 2008; Moore *et al* 2010; Ijas *et al* 2010; Gandhi *et al* 2012). The commonest reason was GI side-effects and less frequent reasons were worsening liver function tests, sepsis, hypoglycaemia, migraine, skin rash, obstetric complications including severe preeclampsia and obstetric cholestasis. The dose of metformin needed to be reduced in 4 studies (the greatest proportion <9%) (Rowan *et al* 2008; Ijas *et al* 2010; Gandhi *et al* 2012; Rai *et al* 2009). The only reported reason was GI intolerance. In contrast, despite high dose of metformin (3000mg), Hassan 2012 study reported to have no GI intolerance.

11. Arthropometric measurements of babies

The largest RCT by Rowan *et al* reported the primary outcome as a composite measure (neonatal hypoglycaemia, respiratory distress, need for phototherapy, birth trauma, 5-minute Apgar score below 7 or premature birth) where they found no significant differences between metformin and insulin treatment (OR 0.99; 95% CI 0.80 to 1.23). They also reported no significant difference on cord blood 'C' peptide levels (but not mention the actual value) and no significant differences in anthropometric measures at birth between the treatments. However, data on anthropometric measures at 2 year follow-up with 50% follow-up rate performed by the same research group (MiG

TOFU) revealed no significant differences in the distribution of fat measured by waist-to-hip ratio and DEXA-calculated abdominal-to-thigh fat ratios but an increased measure of upper-arm circumference and increased thickness of biceps and subscapular skinfolds in toddlers born to metformin-treated mothers (Rowan *et al* 2011). Similarly, Niromanesh 2012 group has observed that significantly lower head, arm and chest circumferences with metformin treatment.

12. Other pregnancy outcomes at birth

There was no significant difference in other neonatal outcomes: respiratory distress, APGAR at 5 minutes, the risks of APGAR <7 at 5 min, serious perinatal outcomes (shoulder dystocia, nerve palsy, fractures, perinatal death), duration of NICU admission, antepartum complications, peripartum complications, postpartum impaired glucose tolerance and maternal hypoglycaemia.

13. Outcomes of follow-up studies

There are three articles which reported the follow-up offspring outcomes of metformin in GDM (Ijas *et al* 2015; Rowan *et al* 2011; Battin *et al* 2015). Data on weight, height and head circumference were available from both studies at toddler follow-up. The meta-analyses showed that children born to metformin-treated GDM women were heavier than those delivered by insulin-treated GDM women. (WMD: 0.44 (95%CI 0.04, 0.84)) kg. There was no difference in height and head circumference. Moreover, Rowan and her group have found that metformin treatment favoured more peripheral deposition of fat than central adiposity. The systolic and diastolic blood pressures of 2-years-old exposed to intrauterine metformin were similar to those of insulin treated women. Moreover, there was no difference in child's motor, linguistic and social development at 18 months of age between metformin and insulin groups.

2.3.2. Comparison 2: Metformin versus Glibenclamide

Table 2.10. Quality assessment of RCTs (Metformin vs Glibenclamide)

Study	Internal Validity										External validity				Potential bias
	Selection		Performance		Detection	Attrition			Reporting	Others					
	Randomization	Allocation concealment	Double Blinding	Same Care pathway	Blinding of outcome assessors	Prior sample size	Intention-to-treat analysis	Acceptable attrition rate	Comprehensive outcome report	Free from other risks of bias	Population	Intervention	Comparator	Explicit reported outcomes	
George 2015	+	+	-	+	+	+	-	-	-	-	+	+	+	+	Low
Moore 2010	+	+	-	+	-	+	+	+	-	+	+	+	+	+	Moderate
Silva 2012	+	?	-	+	-	-	-	-	-	+	+	+	+	+	Low

('+' means appropriate, '-' means not done or not mentioned or not appropriate or '?' means inadequate data to justify that the study)

2.3.2.1 Quality assessment of included studies (Metformin vs Glibenclamide)

Out of three RCTs, randomization and allocation concealment was appropriately conducted except Silva 2012 study where 'brown' envelope, rather than opaque, was used for allocation(Table 2.10). Information on blinding of health care professionals and participants were not reported by any of 3 studies. The diagnostic and target glycaemic levels and management were comparable between metformin and insulin groups in all 3 studies. Only George 2015 mentioned that outcome assessors were blinded from intervention. Although sample size was determined before the conduct of trial, only one study was adequately reported to have done interntion to treat analysis[78]. None of 3 studies reported all outcomes considered important in this review. George 2015 study did interim analysis. The explicit information on population selected, intervention given both on metformin and glibenclamide and the reported outcomes were available in all 3 studies.

2.3.2.2. Characteristic of studies

A brief description on population included, intervention and GDM management were described in table 2.10.

Population: Majority of the GDM being diagnosed by 100g OGTT test. All 3 studies included were RCTs. In all 3 studies, GDM women who failed to achieve target glucose by medical nutrition therapy were randomized. The target glycemic targets were similar across the studies.

Intervention: The maximum dose of metformin was 2000 mg in one study Moore *et al* 2010 and 2500 mg in other 2 studies (Silva *et al* 2013; George *et al* 2015) whereas the maximum dose of glibenclamide was 15mg in one study (Silva *et al* 2013) and 20 mg in other two (Moore *et al* 2010; George *et al* 2015).

Outcomes: Glycemic control was main outcomes in 2 studies and the rest study used composite outcome measures. Data on pregnancy outcomes such as foetal birth weight, caesarean section rates, neonatal hypoglycemia, hypertensive complications of pregnancy, gestational age at birth, maternal fasting and postprandial glycemic control comparing metformin and glibenclamide were available from 3 RCTs.

Table 2.10. Characteristics of included studies (Metformin vs Glibenclamide)

Study ID	Setting (Number)	Population		Care pathway	Metformin	Glibenclamide	Reported side effects necessitating action(n)	Target level	Primary outcomes
		Inclusion	Exclusion						
Moore 2010 (RCT) [152]	United states, University of New Mexico, 75 metformin : 74 glibenclamide	11-33 weeks GA, Screening : 50 g OGCT 1hr ≥130 mg/dl	History of significant renal or hepatic disease, chronic hypertension requiring medication or history of substance abuse	prenatal care provided by university pregnancy diabetic group(OG, diabetes nurse educators and dietiticians. monthly US, twice wkly antenatal testing starting at 28 weeks, elective delivery planned at 38 weeks by IOL or repeat CS, monitored by memory-based glucometer(read within 10% of serum glucose reading),readings correlated with serum glucose each trimester, tested FBS and 2hrPP each meal, compliance accessed by polling the meter and by meeting with diabetes educator at each visit regarding medication use, diet and amount of exercise, weekly reviewed glucose level.	Metformin started at 500 mg/d in divided dose, increased as necessary to maximum 2g/d.	Glibenclamide started at 2.5 mg bd, increased as necessary to maximum of 20 mg/d	GI SE (1)	increased medication if ≥ 2 glucose levels in the same meal exceeded target values by 10 mg/dl/> for 2 consecutive weeks	glycemic control
					Oral medication was discontinued when insulin started. Taking maximum dose of either metformin or glyburide with ≥ 2 glucose levels in the same mean exceeded target values by 10 mg/dl/> for 2 consecutive weeks was considered to be treatment failure.				
Silva 2010, 2012 [122],[191] (RCT)	Brazil, Donal Helena hospital, UNIMED hospital centre, 32 metformin : 40 glibenclamide	GA 11-33 weeks, ≥18 years old, singletons, AC(>10% and < 75%), no maternal and neonatal conditions likely to affect outcomes; High risk group selection by WHO criteria	maternal intolerance to hypoglycaemic medications or unwillingness to participate, foetal AC > 97% or < 5% normal, lack of F-up during pregnancy, malformation on delivery	Multidisciplinary care (nutritionists, physiotherapists, psychologists, nurses, obstetricians, and endocrinologists) met patients every 15 days, monitored by 7 days after instruction, self-assessed FBS and 1hr after breakfast, lunch and dinner using a home capillary glucose monitoring device.	Metformin(Glifage, Diaformin, Dimefor, Glucoformin) 500mg at breakfast and dinner and increased by 500-1000 mg each week until control or maximum of 2500 mg	Glibenclamide(Daonil, Aglucon, Lisaglucon) 2.5 mg before breakfast & dinner and increased by 2.5-5 mg each weeek until control, or to maximum of 20mg/d	Frequent GI complaints, 9 UTI, 7 chronic arterial systemic hypertension	Acceptable upper values (FBS 90 mg/dl and PP 120 mg/dl); if 2 points abnormal, considered impaired.	glucose control, birth weight and neonatal glucose levels
					If not control with maximum dose, replaced by insulin(Insulin 0.7 IU /kg /d (1/3 regular insulin before breakfast, 1/4 regular insulin before lunch & dinner and 1/6 NPH at bedtime.)				

Table 2.10. Characteristics of included studies (Metformin vs Glibenclamide)

Study ID	Setting (Number)	Population		Care pathway	Metformin	Glibenclamide	Reported side effects necessitating action(n)	Target level	Primary outcomes
		Inclusion	Exclusion						
Geroge 2015 (RCT)	South India; large tertiary centre, 79 metformin:80glibenclamide	Women who failed to meet glycaemic targets with medical nutrition therapy(MNT)	Declined to participate; obstetrician declined to allow participation	Home glucose monitoring with glucometer(minimum 4times/week – 1 fasting +3 2hr PP), hypoglycemic events being recorded, regularly checked by research officer	Metformin(glycipha ge) 500mg od, maximum of 2500mg/day	Glibenclamide(Daonil) 2.5 mg given 30 min before meal(started before dinner if uncontrolled fasting; before breakfast if uncontrolled postmeal control); to maximum of 15mg	3 maternal hypoglycaemi a; 1 epigastric burn	glycemic targets-≤5.3mmol/L and 2hr PP ≤6.7mmol/L if maximum dose failed to achieve normoglycemia, within 2-3 weeks they were switched over to insulin	Composite outcome(one of more of outcomes such as macrosomia >3700grams, hypoglycemi a, need for phototherapy , respiratory distress, neonatal death/stillbirt h, birth trauma)
					dose increased in stepwise approach if any two values of fasting ≥6.2mmol/L or PP ≥8.3 mmol/L within a week;				

OGCT – oral glucose challenge test, GA – gestational age, NPH insulin – isophane insulin, GI SE – gastrointestinal side-effects, CS – cesarean section, USG – ultrasound, IOL – induction of labour, UTI – urinary tract infection, GI – gastrointestinal, AC – abdominal circumference, F-up – follow-up, OG – obstetricians and gynaecologists

2.3.2.3. Pregnancy outcomes

Metformin-treated mothers were more likely to deliver babies with lower birth weight than those born to glibenclamide-treated mothers (WMD -44.51 grams [95% CI -98.83, 9.82], n=508, I^2=90%) (Figure 2.10. A). Similarly, the risk of macrosomia was nonsignificantly lower in metformin group (OR 0.57, 0.22, 1.46); I^2=5%) (Figure 2.10.B). The variation in the pooled outcome estimate was attributable to George 2015 study. Among the included RCTs, the George 2015 study had reported GDM with increased triglyceride levels in metformin group. After exclusion this study for sensitivity analysis, the I^2 changed to 0% and the WMD of birthweight -249.13 grams(95%CI -355.88, -142.38) and OR of macrosomia 0.32(95%CI 0.08, 1.21).

Newborns of women who had received metformin developed less neonatal hypoglycemia than those born to glibenclamide-treated GDM women (OR 0.48[95%CI 0.24,0.98];n =508; I^2=61%) (Figure 2.10.C). The large heterogeneity of effect estimates was attributable to the George 2015 study, which included only GDM with moderate hyperglycemia. This might explain exaggeration of metformin effect on the risk of neonatal hypoglycaemia.

The prematurity rate was comparable between metformin and glibenclamide treated GDM [OR 1.59 (95%CI 0.49, 4.80)] (Figure 2.10.D).

The maternal FPG control was better with glibenclamide than metformin with the weighted mean difference(WMD) of 2.40(95%CI 0.21, 4.60); n=508 and I^2=0% whereas there was no significant difference in postprandial glycemic control (WMD: 0.58[95%CI:-2.63, 3.79]; n=508; I^2=0%)(Figure 2.11.A and B).

There was non-significant increased risk of caesarean section with metformin treatment (OR 1.39 [95%CI 0.63, 3.06], n=508, I^2=64%) (Figure 2.11. C) which was largely contributed by Moore 2010 study. In this study, there was a significantly increased in number of metformin-treated women undergoing non-elective caesarean section indicated for non-reassuring fetus.

Maternal weight gain was reported only by Silva 2012 study that there was less maternal weight gain of 2.06 (95%CI 3.98 to 0.14) kg with metformin therapy than glibenclamide in GDM.

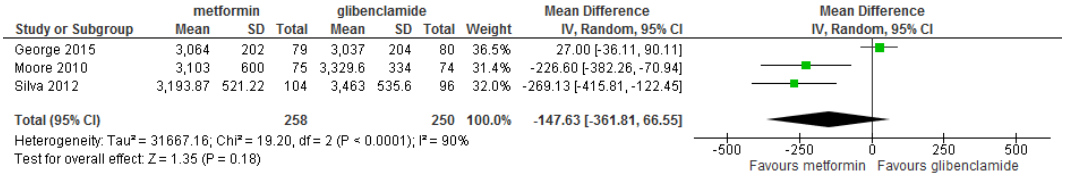
Among 3 studies, the failure rate of metformin was increased in the Moore 2010 study whereas glibenclamide failure rate was higher in the Silva 2012 and George 2015 studies. In Moore 2010 study, the target fasting glucose was 5.8 mmol/L in contrast to 5 mmol/L in other 2 studies. As mentioned above, glibenclamide can have much greater reduction on FPG than metformin. Thus, this study had high metformin failure rate. It should also be noted that in considering therapy failure rate, the maximum dose of the drug should also be taken into account.

There was no significant difference in hypertensive complications of pregnancy, respiratory distress, neonatal jaundice, NICU admission rate, birth trauma and neonatal jaundice.

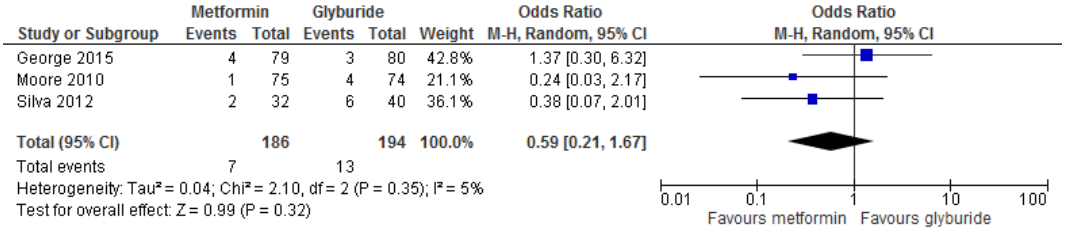
The following outcomes were not reported: small for gestational age, cord blood “C” peptide, neonatal hypocalcaemia, and anthropometric measures and results of postpartum OGTT test.

Figure 2.10. Forest plots of important neonatal outcomes

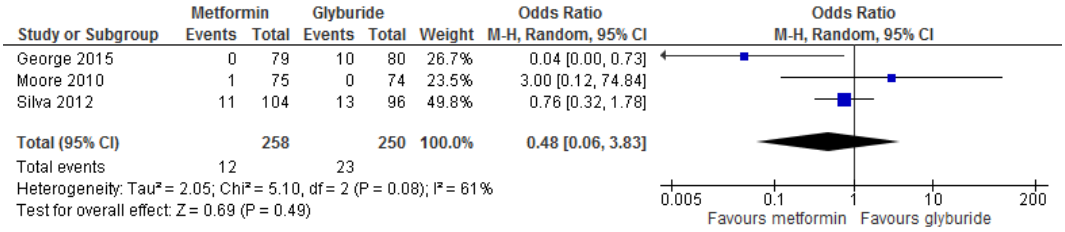
A) Neonatal Birth weight (grams)



B) Macrosomia



C) Neonatal Hypoglycaemia



D) Gestational age at birth (weeks)

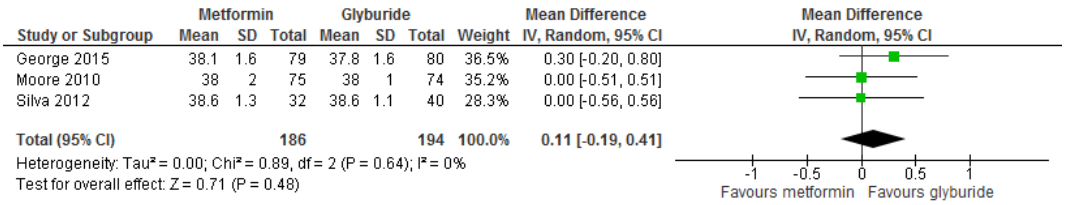
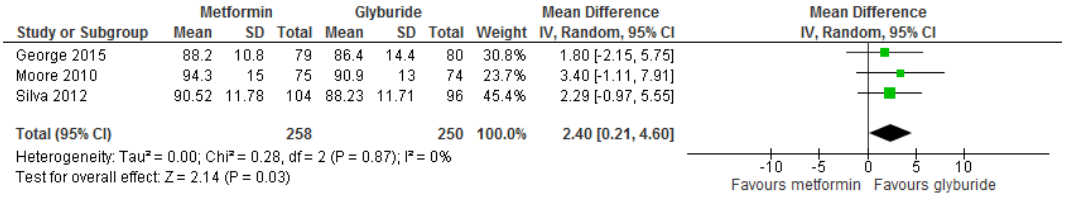
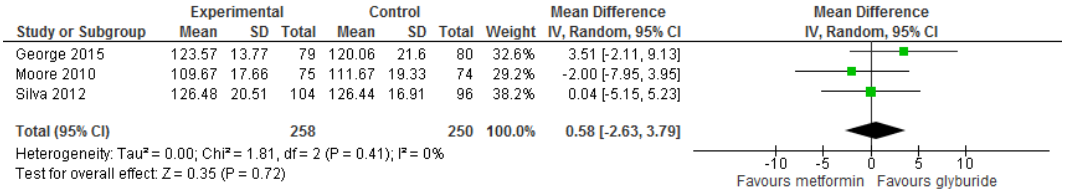


Figure 2.11. Forest plots of important maternal outcomes

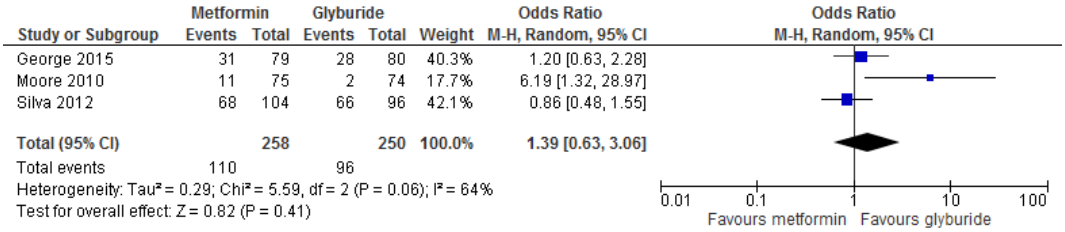
A) Fasting Plasma Glucose Control



B) Postprandial Plasma Glucose Control



C) Caesarean Section Rates



2.3.3. Comparison 3: Metformin vs Diet

Table 2.11. Quality assessment of included studies (Metformin vs Diet)

Study ID	Internal Validity										External Validity				Potential bias
	Selection			Performance		Detection	Attrition		Reporting	Others					
	Consecutive sample	Unbiased Selection Criteria	Baseline Comparability	Same care pathway	Favours group	Blinding of outcome assessors	Prior sample size	Intention-to-treat analysis	Complete outcome report	Confounders controlled	Population	Intervention	Comparator	Explicit reported outcomes	
Tertti 2008	-	-	-	+	D	-	-	-	-	-	+	+	-	+	High
Goh 2012	+	-	-	+	D	-	-	+	-	-	+	-	-	+	High
Balani 2012	+	-	-	+	D	-	-	+	-	-	+	+	-	+	High

('+' means appropriate, '-' means not done or not mentioned or not appropriate or '?' means inadequate data to justify that the study was done appropriately. For baseline comparability and favours group, there were 2 separate items being consider:1) whether the maternal baseline which were considered important in GDM(Age, BMI, parity, glucose levels at OGTT, ethnicity, smoking, previous history of still-birth were considered and reported and 2) if they were unequal across arms, which group could be potentially advantaged out of this biases? D= Diet, I= Insulin, M=metformin, G=glibenclamide, MF= Metformin Failure

2.3.3.1. Quality assessment of included studies

The information on maternal baseline and pregnancy outcome data were available in only 3 observational studies separately. In all three studies, metformin was introduced to GDM women only after diet and lifestyle intervention was failed to meet target glucose levels. Thus, women in diet group were more insulin resistant than those in metformin group which might introduce selection bias. However, all GDM women in each study were treated under the same clinical team and they have similar target glucose levels and pregnancy management. Except Tertti 2008 studies, other 2 studies included all GDM women received diet or metformin within specified time frame were included in analysis. None of the 3 studies have reported comprehensive outcomes for this review and they did not perform any confounder adjustment. For external validity, any of 3 studies did not have enough information on diet strategy.

2.3.3.2. Characteristics of studies

Population: Women in metformin group were found to have significantly higher BMI and higher blood glucose at OGTT as well as be earlier gestational age at OGTT ,less likely to be nulliparous and more likely to have family history of diabetes.

Intervention and Comparator: The maximum dose of metformin (3000mg) was used in Balani 2012 study and obese GDM women in Goh 2012 study. None of the studies reported information on lifestyle modification strategy (i.e diet treatment).

Outcomes: Only Tertti 2008 study described primary outcomes which were birth weight and incidence of neonatal hypoglycaemia.

Table 2.12. Characteristics of included studies (Metformin vs Diet)

Study ID	Intervention		Glucose level at which intervention was started/How each group was formed?		Baseline characters(p<0.01)		
	Diet	Metformin	Diet	Metformin		Diet	Metformin
Tertti[175]2008 (83 diet : 45 metformin)	dietary counselling on low glycemic index and low saturated fat eucaloric diet, dietary record and exercise diary on glucose measurement days kept	Described in Table 2.7	2 out of 3 75g OGTT value(fasting ≥ 4.8, 1hr ≥ 10, 2hr ≥ 8.7 mmol/l)	Fasting ≥5.5 mmol/l at least twice/fasting ≥5.5mmol/l at least once and 90 min PP ≥ 7.8 mmol/l/fasting < 5.5 mmol/l but 90 min PP ≥7.8 mmol/l at least twice within a week of monitoring glucose(4times	At OGTT 0hr(mmol/l) 1hr(mmol/l) 2hr(mmol/l) Hb A1c (%) GA(wk) Primipara n (%)	 5.5±0.3 11±0.9 7.7±1.1 5.5±0.3 27.1±2.4 38(46)	 5.9±0.7 11.7±1.7 8.3±1.8 5.7±0.4 24.8±5.5 10(22)
			Each metformin subject was matched with 2 diet subjects stratified by age and BMI (except 7 metformin, matched by 1:1).				
Goh 2011[187] (371 diet : 465 metformin)	Lifestyle advice and capillary glucose monitoring was initiated.	Described in Table 2.7	Fasting ≥ 5.5 mmol/l (OR) 2hr≥ PP 9 mmol/l at 75g OGTT	Fasting> 5 mmol/l± 2hr PP > 6/6.5 mmol/l within a week of monitoring glucose(4times/day)	Booking BMI (%) <18.5, 18.5-24.9, 25-29.9, ≥30 Ethnicity (%) European Maori Pacific Indian Other Asian Other OGTT(mmol/l) Fasting 2hrPP History of chronic hypertension (%)*	 8.1 48.8 23.7 19.5 30 4.6 7.6 13.5 39.4 5.1 4.5±0.7 9.5±1.1 3.5	 2.2 24 28.5 45.3 22 10.1 20.9 19.1 24.3 3.7 5.3±0.8 9.4±1.6 5.4

Table 2.12. Characteristics of included studies (Metformin vs Diet)

Study ID	Intervention		Glucose level at which intervention was started/How each group was formed?		Baseline characters(p<0.01)		
	Diet	Metformin	Diet	Metformin		Diet	Metformin
Balani 2012 (175 diet:324 metformin)	Individualized dietary advice from a specialist dietician	500mg with meals to maximum of 3G;withdrawn if FGR <10centile and oligohydramnios or reduced end diastolic flow	Fasting > 6 mmol/l (OR) 2hrPP > 7 mmol/l at 75g OGTT	Fasting> 5.6 mmol/l± 1hrPP>8mmol/L± 2hrPP>7mmol/l within a two-week period of monitoring glucose(4times/day)	HbA1c at entry BMI Family history of diabetes	5.4±0.5 27.2±5.8 42(24)	5.6±0.6 30.2±7.1 131(40.4)

GA – gestational age, PP – postprandial, BMI – body mass index, * (p0.001), FGR – Foetal Growth Restriction

2.3.3.3. Results

The summary estimates of the pregnancy outcomes reported by all three studies and analysed in meta-analyses were foetal birth weight, small-for-gestational age, neonatal hypoglycemia, prematurity, NICU admission rates, pre-eclampsia and caesarean section rates.

The pooled risk of small-for-gestational-age was significantly reduced in the metformin group compared to dietary intervention alone (OR 0.63 (95% CI 0.44, 0.89) (n=1478, I^2=0%)). There was no significant difference in the pooled estimates of foetal birth weight (WMD 20.11 [95%CI -39.43,79.65] grams, n=1478, I^2=24%) and large for gestational age(reported by 2 studies).

GDM women receiving metformin were more likely to have induction of labour (OR 1.48 [95% CI 1.04,1.72]; n=627, I^2=0%) and caesarean section (OR 1.35 [95%CI 1.03,1.76], n=1478, I^2=10%). There was marginally significant increase in the pooled risk of neonatal hypoglycemia (OR 1.44 [95% CI 1.0, 2.09]), n=1477, I^2=4%). There was nonsignificant likelihood that metformin groups had increased risk of babies with neonatal jaundice than diet monotherapy (OR 2.78 [95% CI 0.58, 13.02]), n=627, I^2=71%).

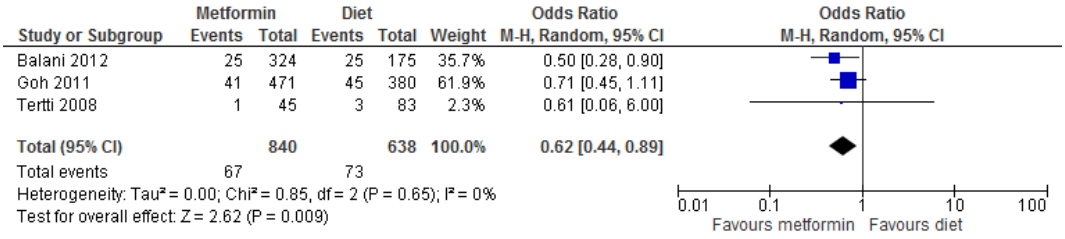
The risks of prematurity, preelampsia and NICU admission rates were not significantly different between metformin and di*et al*one groups.

The possible difference between metformin and diet treatment was observed in the risk of induction of labour with odds of 1.6 (95%CI 0.99, 2.58) in favour of metformin, using random effect meta-analysis (n=524).

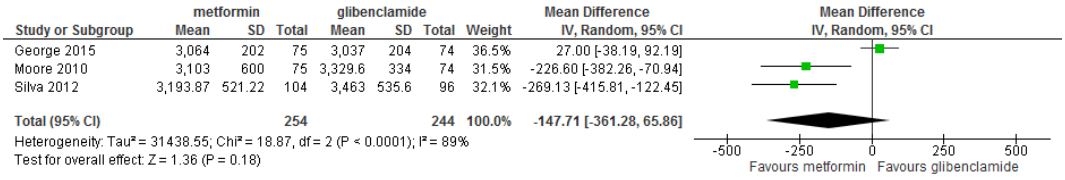
Tertti *et al* reported significant difference in Apgar score at 5 minutes with mean difference of -0.3(95% CI -0.57, -0.03) favoring diet.

Figure 2.12 Forest plots of neonatal outcomes

A) Small for gestational age



B) Neonatal Birth weight



2.3.4.Comparison 4: Metformin Success(metformin alone) vs Metformin Failure(metformin+insulin)

Table 2.13. Quality assessment of included studies (Metformin Monotherapy vs Metformin Failure)

Study ID	Internal Validity										External Validity				Potential bias
	Selection			Performance		Detection	Attrition		Reporting	Others					
	Consecutive sample	Unbiased Selection Criteria	Baseline Comparability	Same care pathway	Favours group	Bliniding of outcome assessors	Prior sample size	Intention-to-treat analysis	Complete outcome report	Confounders controlled	Population	Intervention	Comparator	Explicit reported outcomes	
Ijas 2010	+	-	-	+	M	-	-	+	-	-	+	+	-	+	High
Goh 2011	+	-	-	+	M	-	-	+	-	-	+	-	-	+	High
Tertti 2013	+	-	-	+	M	-	-	+	-	-	+	+	-	+	High
Spaulonci 2013	+	-	-	+	M	-	-	+	-	-	+	+	+	-	High
Corbould 2013	+	-	-	+	M	-	-	+	-	-	+	+	-	-	High

('+' means appropriate, '-' means not done or not mentioned or not appropriate or '?' means inadequate data to justify that the study was done appropriately. For baseline comparability and favours group, there were 2 separate items being consider:1) whether the maternal baseline which were considered important in GDM(Age, BMI, parity, glucose levels at OGTT, ethnicity, smoking, previous history of still-birth were considered and reported and 2) if they were unequal across arms, which group could be potentially advantaged out of this biases? D= Diet, I= Insulin, M=metformin, G=glibenclamide, MF= Metformin Failure

2.3.4.1. Quality of included studies (Table 2.13)

There are 5 studies which presented metformin alone and metformin plus insulin groups separately (Ijas *et al* 2010; Goh *et al* 2011; Tertti *et al* 2013; Spaulonci *et al* 2013; Corbould *et al* 2013). Although three of them were sub study of RCTs, they were qualitatively assessed against NRCTs ROB tools because randomization did not intend for metformin and metformin failure groups. All the women in metformin failure group were received additional insulin when they failed to achieve target glucose levels with maximum dose of metformin. Among all studies, GDM women were more insulin resistant in metformin failure group. All the women who received metformin alone or with supplementary insulin were included for analysis (intention to treat analysis). Neither of NRCTs had blinded the outcome assessors.

2.3.4.2. Characteristics of studies (Table 2.14)

Population: Characters of women who failed to achieve target glucose with metformin were observed in 3 studies.

The RCT performed by Ijäs *et al* reported that metformin failure women were more likely to have BMI ≥ 30kg/m2, more likely to be high risk group of gestational diabetes, had higher fasting glucose level at diagnosis and were more likely to have a history of chronic hypertension compared to the metformin alone treatment group. Similarly, one retrospective observational study undertaken by Goh *et al* observed that the higher the BMI, the greater the proportion of metformin failure; there was significantly higher fasting glucose level (5.7±0.9 vs 5±0.6 mmol/L) and higher proportion of chronic hypertension (8.8% vs 2.4%) in metformin failure group and metformin monotherapy group respectively. Corbould and the group with small

sample size have reported that if fasting glucose at OGTT≤5.2 mmol/L, 13 out of 14 (93%) responded well to metformin (i.e. not require insulin addition). All the women in Tertti 2013 received vitamin supplementation together with metformin.

Data on ethnicity were obtained from 9 studies (Table 2.15). High metformin failure rates of 46.3% and 46.5% were reported by Rowan *et al* and Goh *et al*, respectively. It was found that the study with greater percentages of high risk ethnic groups had metformin failure rate.

Intervention and Comparator: In all 5 studies, metformin failure group was formed when the maximum dose of metformin (ranging from 1500-3000mg) to meet target glucose levels was used. (Tertti *et al* 2013, Ijas *et al* 2010 and Corbould *et al* 2013). Extended release metformin was given in 3 out of 5 studies. In Goh 2011 study, compulsory baseline insulin was given to GDM with fasting ≥ 6 mmol/L at initiation of metformin and the maximum dose of metformin given to obese women were higher than that to normal (3000mg vs 2500 mg). Except Ijas 2010 study where fixed dose strategy of metformin was applied for 3 weeks, the other 4 studies have 1-2 weeks of titration period before insulin was given. In Corbould *et al* 2013 study, insulin was commenced after stopping of metformin compared to insulin addition therapy in other 4 studies.

Outcomes: Neonatal birthweight and macrosomia were primary outcome measure in Tertti 2013 study and Ijas 2010 study respectively whereas Spaulonci 2013 considered glycaemic control for main outcome measure.

Table 2.14. Description of included studies (Metformin monotherapy vs Metformin Failure)

Study ID	How metformin failure group was formed?	Baseline characters		
			Metformin	Metformin + insulin
Ijäs 2010 (32 metformin : 15 metformin +insulin)	If target glucose level (fasting < 5.3 mmol/l and 1.5hr PP <6.7 mmol/l) was not achieved with maximum metformin dose (2350mg) in 1-2 weeks, insulin was added.	Booking BMI* Fasting* 2hrPP GA at metformin initiation*	29.6±5.3 5±0.5 8±1.8 31±3.	35.7±7.2 6.1±1.1 8.7±2.2 26±5.9
Goh 2011 (249 metformin : 216 metformin + insulin)	If fasting ≥ 6 mmol/l, supplemental bedtime isophane insulin was added at the time of metformin initiation. If women failed to achieve target glucose level(fasting 4-5 mmol/l and 2hr PP 4-6/6.5 mmol/l) with maximum metformin dose(2500mg, 3000mg for morbidly obese), they were advised to take supplemental insulin 1/> times/day as required.	Booking BMI (%)* <18.5, 18.5-24.9, 25-29.9, ≥30 Ethnicity (%)* European Maori Pacific Indian Other Asian Other OGTT(mmol/l)* Fasting 2hrPP History of chronic hypertension (%)*	 4.1 33.2 32.8 29.9 21.3 8.4 14.5 21.7 30.9 3.2 5±0.6 9.4±1.4 2.4	 13.5 23.7 62.8 22.7 12 28.2 16.2 16.7 4.2 5.7±0.9 9.3±1.9 8.8
Spaulonci 2013	If target glucose level (fasting < 5.3 mmol/l and 1.5hr PP <6.7 mmol/l) was not achieved with maximum metformin dose in 1-2 weeks, insulin was added.	Booking BMI GA at OGTT*(weeks) HbA1c at OGTT(%) Fasting glucose at OGTT(mg/dL)	29.01±5.73 31.4±2.36 5.82±0.53 98.05±19.03	27.73±5.35 27.55±5.25 6.11±1.17 113.98±26.2
Corbould 2013 (16 metformin : 7 metformin+insulin)	If glucose targets (≤5.0mmol/L fasting ≤6.7 mmol/L at 2hr postprandial) were not achieved with diet/exercise, extended release metformin 500 mg was started with evening meal up to a maximum of 2g over 2 weeks; if then failed, metformin was switched over to insulin.	Fasting glucose at OGTT mmol/L	5.0±0.5	5.6±0.3

AN – Antenatal, * (p0.001), Results described as % or mean ± SD

Table 2.15 Metformin failure rates among different ethnic groups

Study ID (sample size)	Ethnicity %	% of Metformin failure
Rowan 2008	48.2 White **20.1 Polynesian** **10.5 Indian** **13.5 Chinese/Southeast Asia** 7.7 Others	46.3%
Balani 2009	44.9 Caucasian 26 **Asian** 7.9 African	10.2%
Moore 2010	**88 Hispanic** 1 African American 3 Native American 8 White	34.7%
Moore 2007 (Interim result)	63 African American 34 Native American 3 Caucasian	0 %
Goh 2011	22 European **10.1 Maori** **20.9 Pacific** **19.1 Indian** **24.3 Other Asian** 3 Others	46.5%
Gandhi 2012	58.7 White **25.6 Asian** **4.4 Middle-east** 11.3 African	21%
Silva 2012 (approximate)	80 White	25%
Tertti 2008 (approximate)	90 White 10 Arab	18%

2.3.4.3. Results

A total of five studies reported outcomes on metformin failure.

A random effect meta-analysis on 4 studies (n=584) reported that neonates born to women with metformin alone were likely to have lower birth weight than than those to metformin failure mothers with the weighted mean difference of -115.96(95%CI -244.41, 12.50) grams (Figure 2.13.A). Similarly, there was likelihood that the risk of large for gestational age could be reduced in metformin alone therapy group with the odds of 0.60 (95%CI 0.36, 1.07) (Figure 2.13.B)

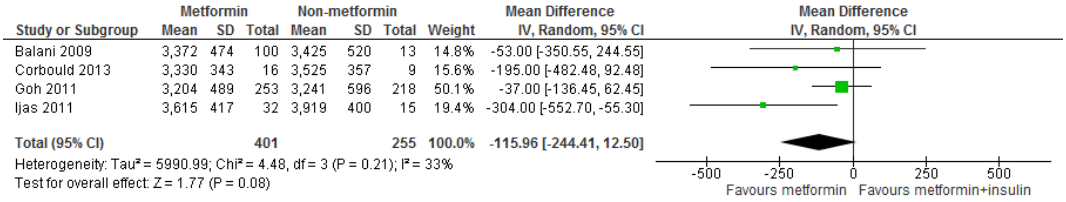
Sensitivity analysis: In Goh 2011 study, basal insulin was introduced at time of metformin initiation if fasting plasma glucose (≥6mmol/L) in comparison to other studies where insulin was only initiated even after maximum dose of metformin failed to reach optimal glycaemic control. Thus, the glycaemic control was expectedly better in Goh 2011 study than other 3 studies and thus the difference in birth weight between two arms was smaller than other studies.

There was no significant difference in risk of macrosomia, small for gestational age, neonatal hypoglycaemia, caesarean section and respiratory distress.

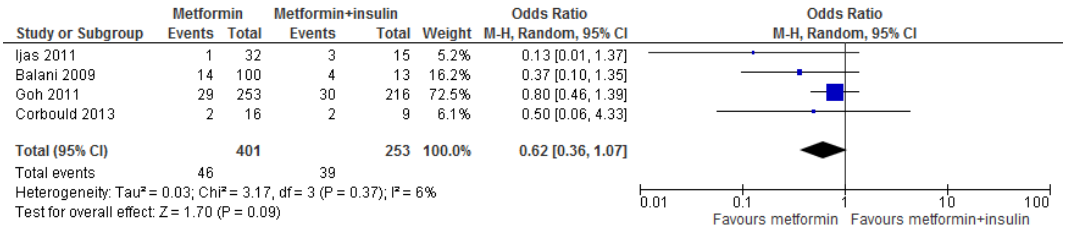
The following outcomes were not reported: risks of induction of labour, weight gain, maternal hypoglycaemia, birth trauma, major or minor congenital abnormality and cord blood C peptide/insulin level.

Figure 2.13. Forest plots of pregnancy outcomes

A) Neonatal Birth weight



B) Large for gestational age



2.4. Discussion

2.4.1 Metformin versus Insulin

All the studies used standard 75g/100g oral glucose tolerance test (OGTT) to diagnose GDM. Similar to previous reports by meta-analyses, we found metformin improved the following outcomes when compared to insulin: maternal weight gain, neonatal hypoglycaemia, NICU admission rates, pregnancy-induced hypertension and heavier weight of 1-2 years old babies (Balsells *et al* 2015; Kitwitee *et al* 2015). However, we did not observe any reduction in risk of pre-eclampsia in our analyses. We also noted a possible reduction in postprandial glycaemia, cesarean section rates, neonatal jaundice and neonatal birth weight, macrosomia and large for gestational age. All other outcomes were comparable between metformin and insulin therapy. Babies born by metformin-treated GDM mothers had earlier gestational age at delivery and they were more likely to be premature. The exact mechanism of metformin-induced prematurity is still unknown and the data on predictors of prematurity among these women are scarce.

The beneficial effect of metformin on maternal weight gain was striking and similar in both RCTs and NRCTs. This is despite the fact that the study by Hassan had high risk of bias (Hassan *et al* 2012). The observed heterogeneity in analyzing the studies might be explained by either the duration of metformin treatment, which was not explicitly reported by any of the included studies; or, the dose of metformin. Our findings agree with the noted beneficial effects of metformin on maternal weight gain in women with PCOS (Vanky *et al* 2010). Our data provides a rationale for using metformin in GDM. As maternal weight gain has been shown to independently predict

LGA, SGA, preeclampsia and caesarean section risks in pregnancy (Kiel *et al* 2007), the use of metformin in GDM may offer additional benefits. Furthermore, as gestational weight gain can strongly predict the metabolic risks of mothers and their babies (Oken et la 2007; Fraser *et al* 2010), it is plausible that metformin can have potential benefit on the long run. There is also evidence that metformin can have better lipid profiles in women and much more favourable fat distribution in the offspring (Niromanesh *et al* 2012; Lord *et al* 2006; Rowan *et al* 2011). Despite significant benefit on maternal weight reduction, there was no increased risk of SGA with metformin use in pregnancy.

In addition to the reduction in maternal weight gain, we noted that metformin use resulted in a possible reduction in post-prandial glucose levels. This is consistent with the findings in type 2 diabetes, where metformin significantly decreases postprandial glucose levels (Howlett *et al* 1999). These decreases in post prandial glucose levels could be ascribed to stimulation of glucagon like peptide-1 (GLP-1) secretion by metformin, which has been noted in animal studies (Yasuda *et al* 2002). Additionally, metformin is known to inhibit dipeptidyl peptidase-IV (DPP-IV) activity in type 2 diabetes patients (Lindsay *et al* 2005). Studies in type 2 diabetes also show a less marked but significant reduction in fasting glycaemia (Howlett *et al* 1999), which we did not observe in our analysis. This may be because the underlying pathology of increased insulin resistance among these two populations are different or metformin may have slower onset of action on fasting glucose levels.

Similar to previous meta-analyses, we also observed a significantly lower incidence of neonatal hypoglycaemia in metformin treated mothers. This was a robust observation noted in both RCTs and NRCTs. This finding might explain the trend of lower

admission to NICU which we also observed. We speculate that the decreased incidence of neonatal hypoglycemia may be due to lower maternal glycemic variability as observed by the more favourable post prandial glucose levels. Another possibility is that the placental transfer of metformin and similar levels of metformin in amniotic fluid means that metformin could be swallowed by intrauterine fetus and helps it adapt to maternal hyperglycemia in the uterine environment.

On the other hand, both RCTs (5RCTs) and NRCTs (4NRCTs) metaanalyses of preeclampsia in our study do not show any difference, which is different from previous analyses. This might be due to difference in definition of pre-eclampsia in systematic review. We separately analysed pregnancy-induced hypertension and preeclampsia. The meta-analysis of PIH including 2 RCTs was found to be different. We have included one more recent RCT in our analyses than the most recent meta-analysis but this did not change any statistical heterogeneity (Kitwitee *et al* 2015).

We saw a beneficial effect of metformin on birth weight, LGA and macrosomia in NRCTs, we did not see this in the RCTs. This may be due to the fact that the population chosen to be given metformin in NRCTs could be influenced by physician's choice. On the other hand, it should be noted that the sensitivity analysis of RCT meta-analyses with exclusion of 2 trials for using extended release metformin have shown significant reduction in birth weight with metformin therapy than insulin. Thus, the effects of metformin on baby's sizes need to be justified by future randomized studies with adequate power to detect the birth weight difference.

In addition to these outcomes, metformin therapy is not only significantly cheaper in terms of drug costs but is also less labour intensive to initiate (Lai *et al* 2008).

Metformin does have a number of side effects, the principal one being GI irritation (Scarpello et apl 2001), whilst insulin is known to be associated with greater incidence of hypoglycemia. Despite these issues, there is evidence that metformin therapy is a more acceptable treatment than insulin (Rowan *et al* 2008).

Our meta-analysis suggests that metformin appears to improve a number of significant clinical outcomes for both mother and baby. More intriguing to us, were those findings in the NRCTs (not replicated in the RCTs) that suggested that metformin was superior to insulin. However, it should be noted that the GDM population who received metformin in NRCTs were not always comparable as in RCTs in that women with higher fasting glucose were added on insulin together with metformin initiation (Goh 2011 study) or they were excluded from metformin group (Tertti 2008 study). It might therefore be expected that the metformin-treated GDM women in NRCTs had better glycaemic control than those in RCTs. We would suggest that future studies should consider these facts when designing the study.

2.4.2 Metformin versus Glibenclamide

Based on three randomized studies, our meta-analysis revealed that metformin and glyburide were likely to have similar effectiveness on GDM outcomes. Although glyburide was found to achieve lower fasting glycemic targets than metformin, there was no difference in important clinical outcomes. On the other hand, metformin-treated GDM mothers were likely to have infants with less neonatal hypoglycaemic risk, lower birth weight and decreased macrosomia than glyburide-treated mothers. These might be possibly related to placental transfer to metformin which is accessible for intrauterine babies and might help them with better handling of hyperglycemic

blood from maternal placental transfer. However, there is possibility of increased non-elective cesarean section rates with metformin, although the number observed was not large enough to draw a valid conclusion. On the other hand, the outcome measure, cesarean section, is criticized to be rather subjective as it could differ with individual caregivers and healthcare settings. In fact, the study was open label study and was carried out in US where glibenclamide was an approved drug in GDM whereas metformin was still in development stage. Thus, it is likely that the clinicians might scrutinize over metformin cases that might increase the increased observation events of non-reassuring fetuses.

We did not find any difference in the treatment failure rate which was found to be one of the factors favoring glibenclamide over metformin in GDM management. The dose of insulin after treatment failure, reported only by Silva *et al*, was not found to be any difference. In fact, if metformin was given to GDM with moderate hyperglycemia and certain ethnic groups like African American, the failure rate was found to be zero (Moore 2010, George 2015). Thus, it would be of great advantage if predictors of metformin responders are identified and metformin is given accordingly.

3.4.3 Metformin versus Diet

Even though baseline characters of metformin treated mothers were found to be higher insulin resistance than those with diet treatment alone, metformin could significantly reduce the risk of small for gestational age than diet monotherapy. Thus, women who are likely to develop foetal growth restriction in late pregnancy in GDM, metformin could be initiated early. Moreover, there is possibility that one trial used metformin at the diagnosis of GDM and they found that early use of metformin could reduce the

dose of insulin requirement without any increase in the risk of adverse pregnancy outcomes over non-metformin group which composed of women on diet or insulin treatment (Gandhi *et al* 2012). On the contrary, one RCT has reported that women who failed metformin needed much higher dose of insulin to meet glycemic targets than those who were already on insulin (Ijas 2010). Hence, if GDM who are at risk of metformin failure are able to be detected early in pregnancy, they can be initiated with insulin medication and thus could save them from unnecessary exposure to higher dose of insulin.

3.4.4 Predictors of metformin failure

All the included studies except Moore2007 study included participants who experienced metformin failure during pregnancy, ranging from 6.7% to 46.5%. Predictors of metformin failure in type 2 diabetes, conducted by Brown *et al*, reported that higher HbA1c, longer history of diabetes and younger age of onset before therapy, were proportionately associated with higher rate of metformin failure (Brown *et al* 2010). In line with this finding in type 2 diabetes, Goh 2011 study, Ijäs 2010 studyand Corbould 2013 study have all reported that metformin failure women were characterized by higher pre-pregnancy BMI and higher glucose levels at diagnosis, indicating higher insulin resistance.

The possible explanation to high metformin failure rate in the MiG trial was suggested by Denice S Feig where he stated that it might be related to greater percentage of high risk ethnic group (Feig 2008). Similarly, in recent study conducted by Farrar *et al*, it was suggested that South Asians were more likely to have lower threshold for insulin resistance than the White (Ferrar *et al* 2015). In our review, the highest failure rates of

metformin was observed in the studies of Rowan *et al* and Goh *et al* with 46.3% and 46.5% respectively. In both studies, the majority (>50%) of samples were high risk ethnicity of gestational diabetes in contrast to <25% of high risk ethnicity in studies done by Balani 2009 study and Gandhi 2012 study, which had low failure rates of 10.2% and 21% respectively. A relatively high failure rate, 34.7%, was observed in the study of Moore 2010 study which was mainly composed of Hispanic population. At the same time, study reported by Gandhi *et al* with almost 60% Caucasian in each group found significant difference in proportion of women with metformin failure where only 21% of treatment failure were observed. Even in high risk ethnic population, we have found that their contribution to the percentage of treatment failure could differ. All in all, we speculated that ethnicity might be a modifiable factor of the potency of metformin in gestational diabetes mothers and it is possible that the failure of metformin may be linked to certain characters of ethnicity.

3.4.5 Metformin Success vs Metformin Failure

The finding of increased proportion of respiratory distress might be probably associated with higher insulin resistance in infants of women with treatment failure. Generally, metformin failure women had increased adverse outcomes than those with metformin alone even though it is still uncertain whether the risk was attributable to impact of either insulin addition or underlying hyperinsulinaemia. In fact, the average dose of insulin after metformin failure reported by 2 studies was around 7-8 units which was significantly higher than average dose of insulin treatment alone (Gandhi *et al* 2012; Ijas *et al* 2010).

3.4.6 Limitation of this review

Regarding effect of metformin compared to insulin, some outcome estimates, like LGA, maternal FPG, SGA and gestational age at birth, were varied between RCT and NRCT results. We should be sceptical in interpretation of evidence from methodologically poor observational studies. In only one out of 6 NRCT (Tertti *et al* 2008), age and BMI were reported to be matched. Neither of other studies considered any confounders in both design and analysis stages. In addition, the representativeness of samples could not be justified as the participants were allocated according to their preference. Although assignment was performed depending on obstetric units in the Rai study, they were managed with different consultants and there was unequal distribution of type 2 diabetes in metformin and insulin arms. Moreover, there were certain cutoff glucose levels for insulin treatment in 2 studies, leading to increased number of more severe GDM in insulin arm. Because of the fact that the robustness of evidence of systematic review is determined by the quality of the included studies, the reliability of this review could be questioned. However, in some cases, the results of NRCT could validate RCT evidence.

At the same time, among RCT, only 4 trials had enough statistical power to correctly investigate the primary outcome. Therefore, methodological limitations of these studies must be taken into account when drawing conclusions. Furthermore, as in NRCTs, lack of blinding of outcome assessors might be a major threat to internal validity. However, most of the outcomes were objective and were defined explicitly. On the other hand, because of a wide range of clinically related operative definitions, the generalizability of effect estimates (e.g. neonatal hypoglycemia) could be upgraded. Likewise, inclusion of diverse ethnic groups across different countries with

relatively interchangeable identification criteria and fairly similar intervention implementation could give further credence to the applicability of the findings.

Specific issues that we considered when performing this analysis included different diagnostic cut-off values, target glucose levels, intervention strategies, primary outcomes considered and definition of outcomes (eg: macrosomia and LGA), some of which differed from study to study. In order to mitigate this, a separate meta-analysis was performed on RCTs and NRCTs. Moreover, the NRCTs had high risk of bias and therefore only outcomes where RCTs alone or RCTs were in agreement with NRCTs have been reported as positive findings.

As regards with conclusions of metformin to glyburide, caution should be taken because of limited number of included studies. However, there were no significant differences in the study quality, design and methods and thus, the combination of these two trials using fixed effect meta-analysis did not result in any conflict findings in-between.

Although we did thorough literature search without limiting language and date, there might be a possibility of publication bias. However, small unpublished studies would probably not alter our conclusions as there was relative similarity in concluded findings in between two included studies. A funnel plot was not adopted as small-study-effect might give a misleading result towards publication bias.

3.4.7 Clinical implications

The clinical aim of GDM management is to have the glycaemic levels within target limits which are reported to be associated with reduction in adverse outcomes of GDM. As metformin can have better and faster postprandial glycaemic control than insulin,

metformin could be a more favourable option for GDM diagnosed with postprandial hyperglycemia. However, there is risk of prematurity with metformin therapy. On the other hand, among studies with moderate hyperglycemia or with supplementary insulin for fasting hyperglycaemia at metformin initiation or with exclusion of metformin failure group from metformin analysis group, prematurity risk was higher in insulin group. Thus, it should be noted that GDM with high fasting hyperglycaemia, insulin or glibenclamide should be given or initiated earlier together with metformin. The glycaemic levels at which additional non-metformin therapy should be added to those risk of metformin failure is not yet determined.

Moreover, compared to insulin, metformin can significantly lower the incidence of maternal weight gain. Thus, among GDM with increased BMI or obese mothers, metformin could be a preferable option to offer. Furthermore, among available GDM medication, metformin is the best anti-diabetic agent on the risk of neonatal hypoglycaemia. This might suggest that metformin can have better reduction in hyperglycemia of both mothers and intrauterine babies. In addition to positive effects on immediate outcomes, the implementation of low-cost, highly efficient metformin as a first-line measure has potential to address the increasing prevalence of GDM and the rising trend of metabolic diseases in the long run. Thus, every GDM mother, who does not have any contraindication to metformin, should receive metformin with appropriate add-on therapy. For this, the treatment algorithm for metformin in GDM is necessary to develop.

At the same time, the current evidence is still limited and in certain outcomes, the benefits of metformin have not been well-established. Therefore, methodologically robust further studies with proper intervention strategy are still necessary. Moreover,

although the data on outcomes beyond pregnancy have been reported, long-term safety of metformin is still needed to be evaluated.

CHAPTER 3

OVERVIEW OF CLINICAL STUDIES

This chapter describes the characteristics of all 299 GDM women included in this thesis which were sub-divided to be used in 2 different clinical chapters namely:

1. Chapter 4 - Predictors of metformin failure in GDM women (STUDY 2)
2. Chapter 5 – Maternal Vitamin B12, Folates and Homocysteine as Determinants of Glycaemia and Birth weight in GDM (STUDY 3)

3.1.Setting

This retrospective review included all consecutive pregnant women with newly diagnosed GDM, according to NICE criteria, attending a GDM clinic in a District General Hospital (George Eliot Hospital, Nuneaton), United Kingdom between January 2009 to December 2012. If the woman had more than one pregnancy during the period, each pregnancy was considered as a separate case. The inclusion and exclusion criteria applied are as follows:

3.2. Study Population

Inclusion criteria

1. Age 18-45 years
2. Singleton pregnancy delivered at GEH hospital
3. GDM newly diagnosed by OGTT

Exclusion criteria

1. Pre-gestational diabetes mellitus (type 1 or 2)
2. History of preeclampsia
3. Medical conditions contraindicated to metformin (like impaired liver and renal function)

4. Women with multiple pregnancies

In this hospital, all high-risk pregnant women (BMI ≥ 30 kg/m2, previous GDM, previous unexplained still birth or baby > 4.5 kg, first degree relative with diabetes, ethnic minority group (South Asians, Middle-Eastern, Afro-Caribbean)) attending antenatal clinic were given 75-grams oral glucose tolerance test (OGTT) between the gestational weeks of 24 and 28 if they were not pre-diabetic. GDM was diagnosed if the fasting glucose of ≥6 mmol/l or/and 2hr postprandial glucose of ≥7.8 mmol/l as per modified WHO 1999 criteria. The GDM clinic was run over this study period, adhering similar GDM management guidelines. All the newly identified GDM were taught by a specialist dietitian and were advised to do home glucose monitoring up to 7 times a day. Capillary blood glucose levels were self-monitored by gluco-meter which were checked regularly and HbA1c levels were accessed every month. The target fasting glucose levels were 4-6 mmol/l and 1hr post meals of < 7.8 mmol/l. All new GDM women were firstly managed with lifestyle modification and if necessary, medication was offered. If 10-20% of either fasting or 2hr postprandial glucose were out of target, the treatment was escalated. The medication used were insulin (intermediate-acting insulin/humulin at night and/or rapid acting insulin with meals) and/or metformin. The allocation of treatment was done by patients agreement upon the use of metformin after consultation. For most women who failed diet and lifestyle treatment, metformin was limited unless rapid control of blood glucose levels were required. The metformin-treated GDM were reviewed after 1 week and titrated against daily blood glucose to the maximum of 2000 mg a day. Metformin was initiated with 500mg once daily at bedtime and increased against glycemic levels up to 2000mg/day. If they failed to achieve target glucose with metformin alone or if they required met,

appropriate insulin was added. And, these metformin-treated GDM women with on top insulin were defined as metformin failure group. Metformin was stopped if there were side-effects.

3.3. Data collection

The data set from PEER study group who collected complete data on pregnancy women from booking to delivery of babies was obtained (Age, BMI, weight in kilograms, parity, ethnicity, smoking status, socioeconomic status, birth weight, gestational age at delivery, mode of delivery, shoulder dystocia, preeclampsia, neonatal intensive care admission). The data set on glucose levels at OGTT during pregnancy and postpartum was obtained from the GEH laboratory. The unique ID was created for each eligible GDM and was matched against these 2 data and combined. The missing data were then completed by manual data collection using pre-defined data collection sheet (appendix 9.2.1). There were very few data on the availability of maternal hypoglycaemia and thus they were excluded in analysis. Data on glycated haemoglobin (HbA1c), vitamin B12, folate and homocystine levels were also recorded from electronic clinical investigation results manually. Then, the data were entered into excel sheet with respective ID. Finally, all the data were combined in one excel spreadsheet.

3.4. Statistical analysis

All statistical analysis were performed by using SPSS version 22.0 (SPSS Inc., Chicago, IL, USA). Non-normally distributed data were log-transformed and appropriate statistical tests were applied. ‘p’ value of <0.05 were considered as statically significant. The detailed analysis for each chapter were described separately in relevant chapter.

3.5. Data Presentation

The categorical variables were described as frequency and percentage and compared using chi-squared test. Based on distribution of data, the continuous variables were presented as mean±standard deviation (normal distribution) and median and interquartile range (non-normal distribution).

3.6. Definition of Clinical Terms used

1) Macrosomia - ≥4000 grams
2) Low birth weight (LBW) - <2500 grams
3) Centiles – calculated using Gestational Related Optimal Weight(GROW) centile chart(adjusting for gestational age, gender, maternal BMI, ethnicity and parity)
4) Large for gestational age(LGA) ->90%
5) Small for gestational age(SGA) - <10%
6) GA – Gestational age
7) OGTT – Oral Glucose Tolerance test
8) MOD – Mode of delivery
9) NSVD – Normal spontaneous vaginal delivery
10) HbA1c – Glycated haemoglobin
11) Premature - <37 weeks
12) Postdate - >42 weeks

13) HbA1c control

1)Good (decrease in HbA1c)

2)Neutral (no change)

3)Not Good (increase in HbA1c)

14) Vitamin B12 (pmol/L)

1)low - <150 pmol/L

2)normal - ≥150pmol/L

15) Folate (ug/L)

1)low - <3.8 ug/L

2)normal – 3.8-16 ug/L

3)high - ≥ 16 ug/L

16) Homocysteine (umol/L)

1)normal - <14umol/L

2)high - ≥14umol/L

Table 3.1 Characteristics of 299 GDM women

Characters(n)	All cases (299)	Diet(31)	Diet+Insulin(46)	Diet+Metformin (62)	Diet+Metformin+ Insulin(89)	Unknown(71)
Age(years)(299)	31(27, 35)	29(25, 32)(31)	31.5(27, 36.3)(46)	30(25, 34)(62)	32(29, 36)(89)	30(27, 34)(71)
Ethnicity(262)						
1) European	226(75.6)	26(83.9)	35(76.1)	47(75.8)	70(78.7)	48(67.6)
2)South Asian	23(7.7)	2(6.5)	3(6.5)	7(11.3)	5(5.6)	6(8.5)
3)Others	13(4.3)	1(3.2)	2(4.3)	1(1.6)	5(5.6)	4(5.6)
Unknown	37(12.4)	2(6.5)	6(13.1)	7(11.3)	9(10.1)	13(18.3)
Parity(254)						
1) Nullip	96(32.1)	11(35.5)	16(34.8)	21(33.9)	28(31.5)	20(28.2)
2) Multip(≥2)	64(21.4)	6(19.4)	10(21.7)	11(17.7)	24(27)	13(18.3)
3) Neither	94(31.4)	11(35.5)	12(26.1)	22(35.5)	25(28.1)	24(33.8)
Unknown	45(15.1)	3(9.7)	8(17.4)	8(12.9)	12(13.5)	14(19.7)
BMI(254)	30.5(26.0,36.3)	27.5(22.8,32.8)	30.1(27.2,33.6)	31.6(27.5,38.0)	31.3(27.6,38.2)	29.3(24.0,36.7)
1)<30	117(39.1)	17(54.8)	18(39.1)	20(32.3)	33(37.1)	29(40.8)
2)≥30	137(45.8)	11(35.5)	20(43.5)	35(56.5)	45(50.6)	22(36.6)
Unknown	45(15.1)	3(9.7)	8(17.4)	7(11.3)	11(12.4)	16(22.5)
Smoking	30(10)	2(6.5)	5(10.9)	6(9.7)	8(9.0)	9(12.7)
Unknown	76(25.4)	7(22.6)	11(23.9)	15(24.2)	18(20.2)	25(35.2)
Weight(223)(kg)	80(68, 97)	73(60.3, 91) (24)	80(73, 91)(35)	84(75, 99)(47)	84(70, 107)(71)	75(62, 94)(46)
Unknown	76(25.4)	7(22.6)	11(23.9)	15(24.2)	18(20.2)	25(35.2)
Treatment type						
1)Diet only	31(10.4)					
2)Diet+Insulin	46(15.4)					
3)Diet+Metformin	62(20.7)					
4)Diet+Metformin+Insulin	89(29.8)					
Unknown	71(23.7)					

Table 3.1 Characteristics of 299 GDM women

Characters(n)	All cases (299)	Diet(31)	Diet+Insulin (46)	Diet+Metformin (62)	Diet+Metformin+ Insulin(89)	Unknown(71)
OGTT (mmol/L)						
1)fasting(299)	4.9(4.5, 5.5)	4.5(4.2,5)	5(4.6, 6.1)	4.6(4.4, 5)	5.2(4.6, 5.8)	4.8(4.3, 5.7)
Missed	0	0	0	0	0	0
2)2hr postprandial(294)	8.5(8, 9.4)	8.1(7.8, 8.8)	4.6(4.4, 5)	8.4(8,9.1)	8.6(8.1, 9.4)	8.5(8, 10.3)
Unknown	5(1.7)	0	1(2.2)	0	2(2.2)	2(2.8)
GA at OGTT(weeks)(223)	26^{+6}(26,29^{+6})	30^{+3}(26^{+5},34)	27^{+5}(26,31^{+6})	26^{+5}(26^{+1},28^{+1})	26^{+3}(22,27^{+4})	27^{+5}(26^{+1},31^{+6})
Unknown	76(25.4)	7(22.6)	11(23.9)	15(24.2)	18(20.2)	25(35.2)
Average HbA1c(220)(%)	5.5(5.3,5.7)	5.3(5.0,5.5)	5.6(5.5,5.9)	5.4(5.2,5.6)	5.5(5.2,5.7)	5.6(5.3,5.9)
Unknown	79(26.4)	14(45.2)	14(30.4)	11(17.7)	12(13.5)	28(39.4)
HbA1c at OGTT(298)(%)	5.5(5.2, 5.7)	5.4(5.1,5.5)(31)	5.6(5.4,5.8)(46)	5.3(5.2,5.6)(62)	5.5(5.3,5.8)(88)	5.5(5.2,5.9)(71)
Unknown	1(0.3)	0	0	0	1	0
Average HbA1c after treatment (221)(%)	5.5(5.2, 5.8)	5.3(5,5.6)	5.7(5.5,6)	5.4(5.1,5.7)	5.5(5.2,5.7)	5.5(5.1,5.9)
Unknown	78(26.1)	14(45.2)	14(30.4)	11(17.7)	11(12.4)	28(39.4)
HbA1c control(220)						
1)Good	105(35.1)	7(22.6)	8(17.4)	24(38.7)	45(50.6)	21(29.6)
2)Neutral	24(8)	3(9.7)	3(6.5)	4(6.5)	8(9)	6(8.5)
3)Not Good	91(30.4)	7(22.6)	21(45.7)	23(37.1)	24(27)	16(22.5)
Unknown	79(26.4)	14(45.2)	14(30.4)	11(17.7)	12(13.5)	28(39.4)
PIH	7(2.3)	0	1(2.2)	2(3.2)	2(2.2)	2(2.8)
PE	3(1)	0	0	0	3(3.4)	0
Unknown	76(25.4)	7(22.6)	11(23.9)	15(24.2)	18(20.2)	25(35.2)
Postnatal OGTT(mmol/L)						
1)Fasting (115)	4.9(4.5, 5.2)	4.4(4.3,4.9)(11)	5(4.6, 5.4)(17)	4.8(4.4, 5.3)(25)	4.9(4.7,5.2)(39)	5.1(4.5, 5.7)(23)
Missed	184(61.5)	20(64.5)	29(63)	37(59.7)	50(56.2)	48(67.6)
2)2hrpostprandial(115)	5.4(4.6, 6.4)	5.4(5.1, 5.5)(11)	5.9(5.2,7.3)(17)	5.4(4.6, 6.4)(25)	5.3(4.5,6.2)(39)	5.7(4.7,7)(23)
Unknown	184(61.5)	20(64.5)	29(63)	37(59.7)	50(56.2)	48(67.6)

Table 3.1 Characteristics of 299 GDM women

Characters(n)	All cases (299)	Diet(31)	Diet+Insulin(46)	Diet+Metformin(62)	Diet+Metformin+ Insulin(89)	Unknown(71)
Birthweight(277)(grams)	3210(2950,3560)	3240(2840,3550)	3225(3042.5,3460)	3190(2928.8,3542.5)	3260(2965,3618)	3180(2885,3580)
1) Macrosomia	18(6)	2(6.5)	0	2(3.2)	5(5.6)	9(12.7)
2) LBW	18(6)	3(9.7)	2(4.3)	3(4.8)	6(6.7)	4(5.6)
3) Normal	241(80.6)	22(71.0)	42(91.3)	51(82.3)	75(84.3)	51(71.8)
Unknown	22(7.4)	4(12.9)	2(4.3)	6(9.7)	3(3.4)	7(9.9)
GA at delivery(weeks)(283)	$38^{+3}(38^{+0},38^{+6})$	$38^{+6}(38^{+0},39^{+4})$	$38^{+3}(38^{+0},38^{+6})$	$38^{+3}(38^{+0},38^{+6})$	$38^{+1}(37^{+1},38^{+4})$	$38^{+4}(38^{+0},39^{+4})$
1)premature	34(11.4)	2(6.5)	4(8.7)	5(8.1)	20(22.5)	3(4.2)
2)normal	248(82.9)	27(87.1)	40(87)	52(83.9)	66(74.2)	63(88.7)
3)postdate	1(0.3)	0	0	0	0	1(1.4)
Unknown	16(5.4)	2(6.5)	2(4.3)	5(8.1)	3(3.4)	4(5.6)
Centiles (272)	42.8(17.0,74.7)					
1)SGA	41(13.7)	6(19.4)	4(8.7)	8(12.9)	10(11.2)	13(18.3)
2)Normal	203(67.9)	19(61.3)	36(78.3)	43(69.4)	63(70.8)	42(59.2)
3)LGA	28(9.4)	2(6.5)	3(6.5)	3(4.8)	12(13.5)	8(11.3)
Unknown	27(9)	4(12.9)	3(6.5)	8(12.9)	4(4.5)	8(11.3)
MOD(222)						
1)NSVD	122(40.8)	13(41.9)	23(50)	29(46.8)	35(39.3)	22(31)
2)Assisted	21(7)	5(16.1)	3(6.5)	5(8.1)	7(7.9)	1(1.4)
3)CS	79(26.4)	6(19.4)	9(19.6)	12(19.4)	29(32.6)	23(32.4)
i)Emergency	33(41.8)	3(50)	3(33.3)	4(33.3)	12(41.4)	11(47.8)
ii)Elective	45(57)	3(50)	6(66.7)	8(66.7)	16(55.2)	12(52.2)
iii)Unknown	1(1.3)	0	0	0	1(3.4)	0
Unknown	77(25.8)	7(22.6)	11(23.9)	16(25.8)	18(20.2)	25(35.2)
NICU admission(195)	1(0.3)	0	0	1(1.6)	0	0
Unknown	104(34.8)	9(29)	17(37)	20(32.2)	24(27)	34(47.9)
Shoulder dystocia(153)	3(1)	0	0	2(3.2)	1(1.1)	0
Unknown	146(48.8)	17(54.8)	25(54.3)	28(45.2)	33(37.1)	43(60.6)

Table 3.1 Characteristics of 299 GDM women

Characters(n)	All cases (299)	Diet(31)	Diet+Insulin(46)	Diet+Metformin (62)	Diet+Metformin+ Insulin (89)	Unknown(71)
GA at B12(weeks)(174)	$28^{+1}(25^{+6},30^{+5})$	$30^{+1}(27^{+5},34^{+1})$	$29^{+1}(27,32^{+1})$	$28(25^{+6},30^{+6})$	$27^{+2}(21^{+2},29^{+5})$	$28^{+5}(26^{+7},33^{+4})$
Unknown	125(41.8)	18(58.1)	21(45.7)	20(32.3)	21(23.6)	45(63.4)
Vitamin B12 (207) pmol/L	165.3(136.5,212.5)	187.5(136.5,218.4)	169.7(134.3,212.5)	156.8(132.8,214.9)	163.8(134.7,201.5)	170.9(144.7,215.1)
1)low	64(21.4)	4(12.9)	11(23.9)	15(24.2)	26(29.2)	8(11.3)
2)normal	143(47.8)	11(35.5)	16(34.8)	35(56.5)	55(61.8)	26(36.6)
Unknown	92(30.8)	16(51.6)	19(41.3)	12(19.4)	8(9)	37(52.1)
Folate(188) ug/L	9.7(6.8, 13.4)	10.4(7.7,16.5)	9(6,15.6)	10(5.5,13.4)	9.7(6.8,13.3)	9.8(7,13.2)
1)low	6(2)	2(6.5)		3(4.8)	1(1.1)	
2)normal	150(50.2)	8(25.8)	20(43.5)	36(58.1)	61(68.5)	25(35.2)
3)high	32(10.7)	4(12.9)	6(13)	6(9.7)	12(13.5)	4(5.6)
Unknown	111(37.1)	17(54.8)	20(43.5)	17(27.4)	15(16.9)	42(59.2)
Hcy(115) umol/l	5.7(4.9, 7)	5.2(5.8)	6(5,7)	6(4.75, 8)	5(4,7)	5.5(4,7)
1)Normal	114(38.1)	9(29)	12(26.1)	30(48.4)	43(48.3)	20(28.2)
2)High	1(0.3)				1(1.1)	
Unknown	184(61.5)	22(71)	34(73.9)	32(51.6)	45(50.6)	51(71.8)

CHAPTER 4

PREDICTORS OF METFORMIN FAILURE

IN GESTATIONAL DIABETES MELLITUS

(STUDY 2)

4.1.Introduction

Based on our systematic review in earlier chapter, it is evident that metformin is clearly shown to be superior to insulin in some pregnancy outcomes, whereas glyburide is found to be inferior to metformin in GDM (Balsells *et al* 2015). Moreover, women preferred metformin over insulin. However, there are up to 50% of metformin-treated GDM that are at risk of requiring additional insulin for optimal glycaemic control (referred as metformin failure). If these women are able to be identified at GDM diagnosis, it will enable healthcare professionals to target these women with much more stringent glycaemic control (i.e. metformin given together with appropriate insulin). Consequently, the better glycaemic control can reduce the related adverse outcomes of GDM, while the effect of metformin on less maternal weight gain might help pregnancy outcomes. Hence, it could result in overall improvement in GDM management.

The studies on the maternal characters in relation to metformin failure are still very limited. Some studies have reported that metformin-treated women with GDM who needed insulin have higher BMI and are possibly more insulin resistant (higher fasting & postprandial glucose and fructosamine at diagnosis)(Tertti *et al* 2013, Ijas *et al* 2010, Goh *et al* 2011. Spaulonci *et al* 2013). One small study reported that GDM with fasting glucose at OGTT ≤5.2 mmol/l can achieve optimal glycaemic levels with metformin alone (Corbould *et al* 2013). Moreover, it could be assumed that being European ethnicity have lower risk of metformin failure than Hispanic or South Asians (Moore *et al* 2010; Rowan *et al* 2008). Recently, one small study on predictors of metformin failure has suggested that if fasting glucose levels at OGTT is greater than 5.2 mmol/L, the response to metformin is decreased by two-thirds (Corbould *et al* 2013). Thus, it

is possible that metformin response is reduced in GDM women with increased insulin resistance at enrolment.

Being slow-onset in action, it can be hypothesized that if metformin is given as soon as after GDM is diagnosed, the response rate might be better and its benefit on maternal weight gain could be enhanced. In addition, one cohort study which compared metformin to diet therapy in GDM has reported that metformin has a beneficial effect on the rates of macrosomia and small-gestational-age (SGA) despite inclusion of increased number of more hyperglycemic and obese women in metformin group (Balani *et al* 2012). This might indicate that metformin initiation at diagnosis could improve GDM outcomes. Furthermore, it would also be helpful for healthcare professionals in remote areas to identify the GDM cases which might need referral to tertiary centre for insulin treatment. This could further save healthcare resources.

Clearly, it is necessary to establish a GDM treatment algorithm with metformin. Therefore, we carried out a study to identify maternal characters that can predict metformin failure in GDM.

4.2.Methods

4.2.1 Study population and data collection

All the GDM who received metformin in our centre during the study period were eligible. They were included if they received metformin for at least 2 weeks, continued metformin until delivery and delivered a single live baby at this hospital. GDM diagnosis and management has already described in chapter 3.

In this centre, metformin was given as an alternative option to GDM women who failed diet and lifestyle treatment. Metformin was offered as first-line unless rapid control of blood glucose levels was required. The metformin-treated GDM were reviewed after 1 week and titrated against daily blood glucose to the maximum of 2000 mg a day. If they failed to achieve target glucose with metformin alone, either intermediate-acting insulin (humulin) at night or rapid-acting insulin with meals were added accordingly. Metformin was stopped if there were side-effects.

Demographic characters and all pregnancy and birth data including HbA1c throughout pregnancies were recorded. The outcome variables considered were: birth weight (BWt), macrosomia (BWt≥4000grams), low birth weight (LBW) (BWt<2500 grams), centiles using GROW (Gestation related optimal weight) chart, Large-for-gestational-age(LGA)(>90centiles), SGA(<10centiles), gestational age at delivery, prematurity(<37 weeks), APGAR at 1 and 5 minutes, Mode of delivery, Shoulder dystocia, Preeclampsia, NICU admission rates, Total duration of metformin, postnatal fasting and 2hr postprandial and HbA1c during treatment. Metformin failure group was defined as metformin-treated GDM women with add-on insulin therapy, whereas

metformin monotherapy group was defined as GDM women with metformin alone treatment.

4.2.2 Statistical analysis

Statistical analysis were performed using SPSS programme version 22.0 (SPSS Inc., Chicago, IL, USA). Skewed data were log-transformed and analysed as below. Continuous data were described in mean±standard deviation (SD) and categorical data were described in number (n) and percentages(%). P value of 0.05 was considered as statistically significant.

4.2.2.1 Question being addressed

1. What are the maternal characters associated with metformin failure?

   Statistical model applied to compare the maternal characters between metformin failure and metformin monotherapy groups:

   i) Log-transformed data were used in independent 't' tests to examine the difference in maternal characters (Age, BMI, fating glucose at OGTT, 2hr postprandial glucose at OGTT, HbA1c at OGTT, GA at OGTT, GA at metformin initiation)

   ii) Chi-squared tests to examine the difference in the percentages of ethnicity and smoking status

2. Are pregnancy outcomes of GDM women with metformin failure comparable to those with metformin monotherapy?

   Statistical model applied to compare the pregnancy outcomes between metformin failure GDM group and metformin monotherapy GDM group

i) Log-transformed data were used in independent ‘t’ tests to examine the difference in pregnancy outcomes (birth weight, centiles, gestational age at delivery, APGAR scores at 1 and 5 minutes, duration of metformin, average HbA1c during treatment, fasting glucose at postnatal OGTT and 2hr postprandial glucose at postnatal OGTT)

ii) Chi-squared tests to examine the difference in the percentages of babies with macrosomia, , LBW, LGA, SGA and prematurity, Caesarean section rates (Elective and Emergency), Shoulder dystocia and NICU admission rates

3. What is the strongest maternal character to predict metformin failure?

   Statistical model applied:

   Receiver-Operator Curve (ROC) to examine the areas under curve (AUC) of the four significant maternal characters identified in question (1) which are in match with previous studies (age, fasting glucose at OGTT, HbA1c at OGTT, GA at dietary failure) to predict metformin failure and to establish the value with best sensitivity and specificity

4. What is the best criterion to identify GDM women with high risk of metformin failure in current study?

   Statistical model applied:

   Regression model to determine the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the maternal characters that can best identify the GDM with metformin failure

5. Are there any difference in the outcomes between metformin failure GDM and insulin treated GDM women?

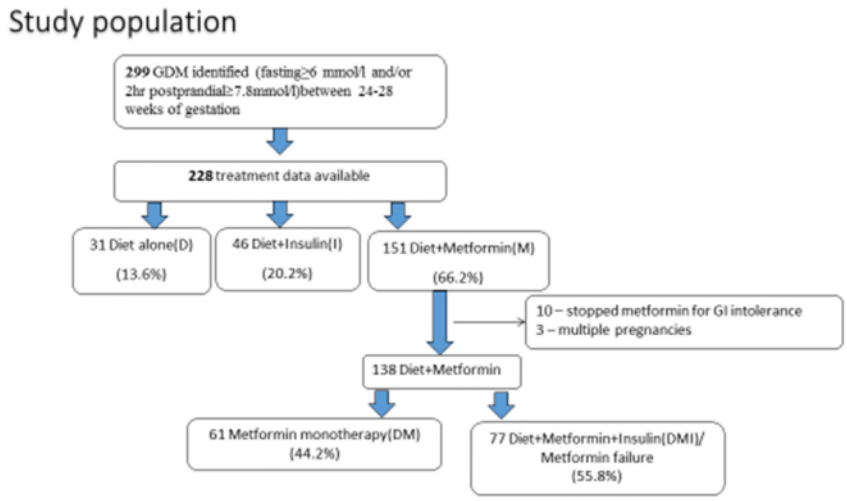
   Statistical model applied to compare the pregnancy outcomes between metformin failure GDM group and insulin treated GDM group

   i) Log-transformed data were used in independent ‘t’ tests to examine the differences in pregnancy outcomes (continuous variable mentioned in item (1))

   ii) Chi-squared tests to examine the difference in the percentages of categorical outcome variable stated in item (1))

4.3.Results

Figure 4.1: An overview of participants included in the study



4.3.1 Characteristics of GDM women included

During the study period, there were 299 GDM identified, data on 71 women were failed to record in the chart. Out of 228 GDM women with available treatment data, 197(86.2%) GDM women on diet needed additive therapy. 151(66.2%) received metformin as first-line. Among metformin-treated GDM women, three were multiple pregnancies and 10 stopped metformin before delivery for GI intolerance and changed to insulin, thus, they were excluded from the analysis. Multiple pregnancies were excluded from both mother and infant outcomes because hormonal disturbances due to increased placental size in multiple pregnancies can increase the insulin resistance of mothers and also they can also interfere with infant birth size. Of the included 138 metformin-treated GDM, 77(55.8%) needed supplementary insulin (metformin failure). The starting dose of metformin was 500 mg od, the maximum dose was 2000 mg and the median dose at delivery was 1500mg.

4.3.2 Metformin monotherapy vs Metformin Failure:

The maternal characters and outcomes of GDM on metformin monotherapy were compared with metformin failure group.

4.3.2.1. Maternal baseline characteristics of GDM women with metformin monotherapy and metformin failure

The baseline characteristics of GDM women between the metformin failure and metformin success groups were compared in Table 4.1. There was no difference in pre-pregnancy body mass index (BMI), parity, ethnicity, smoking status and 2hr postprandial glucose at OGTT. The average age of GDM women with metformin failure group were older (30.0 vs 32.4years, $p<0.001$). They were more likely to have higher fasting glucose (4.83 vs 5.32mmol/L, $p<0.05$) and HbA1c at OGTT (5.38 vs 5.32%, $p<0.05$) as well as GA at OGTT (27 vs 25 weeks, $p<0.05$) and GA at dietary failure/metformin initiation (29^{+3} vs 26^{+2} weeks) (Table 4.1).

Table 4.1. Maternal baseline characteristics of GDM with metformin monotherapy vs metformin failure

Characters	**Metformin monotherapy (61)** Mean±SD; n(%)	**Metformin Failure (77)** Mean±SD; n(%)	**Mean Difference/Odds Ratio(95% CI)**
Age(years)	30±5.9	32.4±5.5*	-2.3(-4.22, -0.38)
BMI	32.4±7.1	32.7±7.1	-0.27(-2.83, 2.30)
Ethnicity European South Asian	 46(75.4) 7(11.5)	 60(77.9) 3(3.9)	
Parity Nullip Multip(≥2)	 21(34.4) 11(18)	 23(29.9) 22(28.6)	 1.15(0.50, 2.66) 2.10(0.82, 5.36)
Smokers	6(9.8)	7(9.1)	0.87(0.27, 2.78)
GA at OGTT(weeks)	$27\pm5^{+0}$	$25\pm6^{+1}$	1.1(1.0, 1.19)days
OGTT Fasting(mmol/l) 2hr postprandial(mmol/l)	 4.83±0.66 8.59±1.18	 5.4±0.92 8.87±1.5	 -1.1(-1.15, -1.05) -1.02(-1.07, 1.02)
HbA1c at OGTT(%)	5.4±0.33	5.6±0.48	-1.05(-1.07, -1.01)
GA at metformin initiation (weeks)	$29^{+3}\pm4^{+0}$	$26^{+2}\pm6^{+4}$	1.12(1.1, 1.2)days

4.3.2.2. Pregnancy outcomes of GDM women with metformin monotherapy and metformin failure

The pregnancy outcomes were similar between the two groups except gestational age at delivery (Table 4.2). HbA1c levels during treatment were available only in 84.1%(116) after OGTT/during metformin treatment of GDM women and the glycemic levels were similar between the groups. Metformin failure mothers delivered babies at earlier gestational age (-1.02 (95%CI -0.29, -1.01) days and the risk for prematurity was significant (OR: 3.24(1.01, 10.39) (Table2). Weights and centiles of babies born to GDM women with metformin failure were similar to those on metformin alone therapy. There was no difference in the percentages of other neonatal outcomes such as caesarean section rate, neonatal intensive care admission rate, APGAR scores at 0min and 5min atdelivery and sholulder dystocia.

Table 4.2: Pregnancy outcomes between metformin monotherapy and failure

Outcomes	**Metformin monotherapy (61)**Mean±SD; n(%)	**Metformin Failure (77)** Mean±SD; n(%)	**Mean Difference/Odds Ratio (95% CI)**
Birthweight(grams)	3217.4±533.3	3265.8±496.8	-48.39(-228.98, 132.2)
Macrosomia(≥4500 grams)	2(3.8)	4 (5.2)	1.54(0.27, 8.74)
LBW(<2500 grams)	3(5.7)	5(6.5)	1.28(0.29, 5.62)
Centile	42.08±27.28	53.0±32.3	-10.87(-21.97, 0.23)
LGA(>90%)	3(6.5)	10(13)	2.61(0.68, 10.06)
SGA(<10%)	7(11.5)	8(10.4)	0.89(0.30, 2.66)
GA at delivery(weeks)	$38^{+2}\pm1^{+3}$	$37^{+5}\pm1^{+5}$	3.96(0.07, 7.86)days
Prematurity(<37 weeks)	5(8.9)	15(19.5)	3.24(1.01, 10.39)
APGAR at 1 minute	8.6±1.0	8.6±0.97	0.03(-0.34, 0.41)
APGAR at 5 minutes	9.0±0.5	9.1±0.38	-0.10(-0.27,0.06)
Caesarean section	12(19.7)	26 (33.8)	2.05(0.89, 4.69)
Emergency	4	11	
Elective	8	14	
Shoulder dystocia	2(5.9)	1(1.3)	0.96(0.88, 1.06)
Preeclampsia	0	3(3.9)	1.05(0.99, 1.11)
NICU admission	1(1.6)	0	0.98(0.93, 1.02)
HbA1c after OGTT(%)	5.45±0.42	5.5±0.46	-0.20(-0.34, -0.06)
Duration of metformin(days)	63.11±28.28	78.98±40.62	-15.87(-30.35, -1.38)
Postnatal OGTT	n=25	n=35	
Fasting	4.8(4.4,5.3)	5.0±0.51	-0.21(-0.47, 0.05)
2hr postprandial	5.6±1.5	5.4±1.5	0.23(-0.57, 1.02)

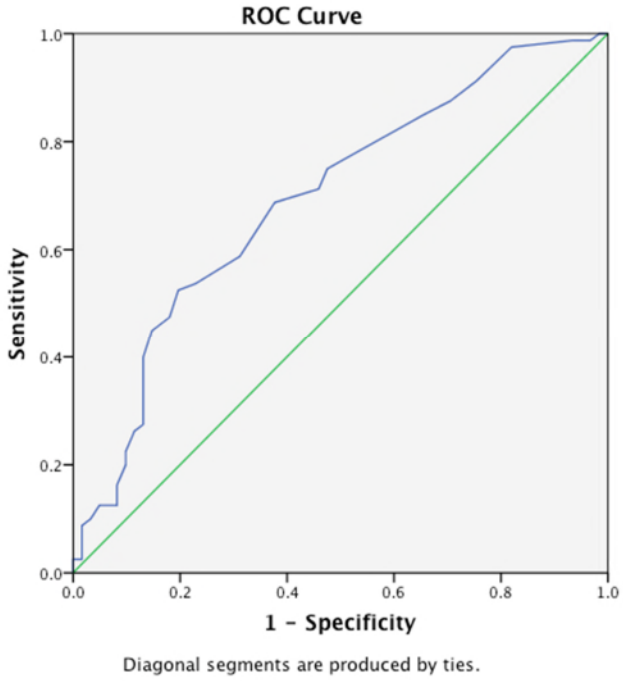
NS = Not significant; SD=standard deviation; CI= confidence interval; n=number

4.3.2.3.Predictors of metformin failure in GDM women

A number of different cut-off points for 4 significant maternal characters(age, gestational age at OGTT, fasting at OGTT and HbA1c at OGTT) between metformin failure and metformin monotherapy were accessed in receiver-operator curve (Figure 5.2) and the best predictive cut-off value was determined(Table 4.3 & 4.4). The best stand-alone predictors for screening were fasting level at OGTT>4.8 mmol/l(sensitivity 69%, specificity 62%), age≥30 years at booking (sensitivity 71.4%, specificity 47.5%) and HbA1c at OGTT >5.5%(sensitivity 71.1%, specificity 52.5%). If the fasting level of ≥5.1 mmol/l (IADPSG fasting criteria) was used, the positive predictive value of metformin failure was 78%. If the criteria 1) fasting glucose at OGTT >4.8 mmol/l OR 2) fasting glucose at OGTT ≤4.8mmol/l & gestational age at dietary failure≤27^{+5} were used, the metformin failure was predicted with a sensitivity of 87% and specificity of 64% and a positive predictive value of 74% and a negative predictive value of 70%. We applied this criteria in our population (Table 4.5).

Figure 4.2 Receiver Operator Curve (ROC) of potential predictors of metformin failure

A) Fasting glucose levels at OGTT



B) Gestational age at OGTT

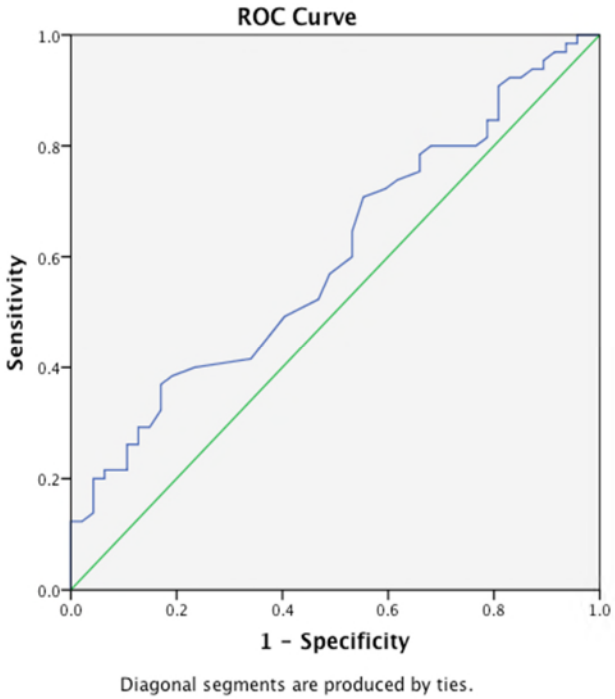


Table 4.3: Area Under Curve (AUC) of maternal characters of metformin failure

Maternal Predictors	**AUC(95% Confidence Interval)**	**p value**
1. Age at booking	60.4 (50.8-69.9)	0.037
2. GA at dietary failure	32.6 (22.5-42.7)	0.002
3. Fasting level at OGTT	69.4 (60.7 - 78.2)	<0.001
4. HbA1c at OGTT	62.8(53.8-72.2)	0.01

Table 4.4: Characteristics Predictive of metformin failure

Criteria	**Sensitivity**	**Specificity**	**PPV**	**NPV**
1. Age≥30 years at booking	71.4%	47.5%	63%	57%
2. Fasting OGTT > 4.8 mmol/l	69%	62%	71%	60%
3. Fasting OGTT ≥ 5.1 mmol/l(IADPSG fasting criteria) ≥ 5.6 mmol/L(NICE 2015 Fasting criteria)	53% 35%	80% 87%	78% 77%	56% 50.1%
4. GA at dietary failure ≤ 27^{+5} weeks	50%	80%	78%	54%
5. Fasting OGTT > 4.8 mmol/l, OR ≤ 4.8 & GA at dietary failure ≤ 27^{+5} weeks	88%	85%	63%	70%
6.HbA1c at OGTT >5.5%	7.1%	52.5%	65.1%	59.3%

Table 4.5: Criteria for predicting metformin failure after dietary failure

	Fulfils criteria		
Metformin group	Yes	No	Total
Metformin success	40	21	61
Metformin failure	68	9	77
Total	108	30	

GDM diagnosed when 1) fasting at OGTT≥4.8mmol/l or 2) fasting at OGTT<4.8 mmol/l but gestational age at dietary failure was ≤27^{+5} weeks. p value for this table calculated by x^2 test is <0.05.

4.3.2.4.Pregnancy outcomes of GDM women with metformin plus insulin (metformin failure) and insulin alone therapy

Compared to insulin alone therapy group, women in metformin plus insulin group were diagnosed GDM at later gestational age by average of 25.5 days(Table 4.6 and Table 4.7). However, their glycaemic control after diagnosis of GDM were lower than those with insulin monotherapy. The other baseline characteristics including fasting and postprandial glucose levels at OGTT were comparable between the two groups. After adjusting for gestational age at OGTT , there was still significant difference in average HbA1c levels between insulin alone and metformin failure groups ($p<0.01$). There was no difference in pregnancy outcomes between women with metformin failure and those with insulin alone therapy.

Table 4.6. Baseline characteristics of GDM women between metformin failure and insulin alone groups

Characters	**Metformin Failure (77)** Mean±SD; n(%)	**Insulin Alone (45)** Mean±SD; n(%)	**Mean Difference/Odds Ratio(95% CI)/'p'value**
Age(years)	32.4±5.5	31.7±5.9	0.64(-1.46,2.74)
BMI	32.7±7.1	30.9±6.1	1.81(-0.92,4.53)
Ethnicity			
European	60(77.9)	35(77.8)	NS
South Asian	3(3.9)	3(6.7)	NS
Parity			
Nullip	23(29.9)	16(35.6)	NS
Multip(≥2)	22(28.6)	10(22.2)	NS
Smokers	7(9.1)	5(11.1)	NS
GA at OGTT(weeks)	$25^{+0}\pm5^{+3}$	$28^{+4}\pm4^{+5}$	-25.5(-40.7,-10.32)days
OGTT			
Fasting(mmol/l)	5.4±0.92	5.3±1.0	0.02(-0.33,0.37)
2hr postprandial(mmol/l)	8.87±1.5	9.2±1.64	-0.28(-0.86,0.29)
HbA1c at OGTT(%)	5.6±0.48	5.63±0.44	-0.28(-0.20,0.14)

Table 4.7. Pregnancy outcomes of GDM women between metformin failure and insulin alone groups

Outcomes	**Metformin Failure (77)** Mean±SD; n(%)	**Insulin Alone (45)** Mean±SD; n(%)	**Mean Difference/Odds Ratio (95% CI)/'p' value**
Birthweight(grams)	3265.8±496.8	3231.3±388.2	NS
Macrosomia(≥4500 grams)	4 (5.2)	0(0)	NS
LBW(<2500 grams)	5(6.5)	2(4.4)	NS
Centile	53.0±32.3	46.1±27.4	6.88(-4.78, 18.53)
LGA(>90%)	10(13)	3(6.7)	2.09(0.54, 8.03)
SGA(<10%)	8(10.4)	4(8.9)	1.19(0.34, 4.19)
GA at delivery(weeks)	$37^{+5}\pm1^{+5}$	$38^{+2}\pm1^{+0}$	-4.24(-7.56,-0.92)days
Prematurity(<37 weeks)	15(19.5)	3(6.7)	3.39(0.92, 12.43)
APGAR at 1 minute	8.6±0.97	8.7±0.73	-0.09(-0.47,0.28)
APGAR at 5 minutes	9.1±0.38	9.1±0.37	-0.004(-0.16, 0.16)
Caesarean section	26 (33.8)	9 (20)	1.31(0.97, 1.78)
i)Emergency	11(14.3)	3(6.7)	
ii)Elective	14(18.2)	6(13.3)	
Shoulder dystocia	1(1.3)	0	NS
Preeclampsia	3(3.9)	0	NS
NICU admission	0	0	NS
HbA1c after OGTT (%)	5.5±0.46	5.8±0.42	-0.31(-0.50, -0.12)
Postnatal OGTT	n=35	n=17	
Fasting	5.0±0.51	5.2±0.95	-0.20(-0.61, 0.20)
2hr postprandial	5.4±1.5	5.9±1.7	-0.49(-1.45, 0.46)

NS = Not significant; SD=standard deviation; CI= confidence interval; n=number

4.4 Discussion

This chapter observed that over three-fourths (76.7%) of GDM women chose metformin as their first-line therapy in our centre between 2009 and 2012. Over 80% (127 out of 151) metformin-treated GDM women were diagnosed as GDM by higher post-prandial hyperglycaemia alone. Metformin has been included in NICE GDM guidelines as second choice (i.e. with women's consent) since 2007. It is in accordance with MiG trial which reported that women preferred metformin over insulin (76.6% vs 12.6% respectively) (Rowan *et al* 2008). Moreover, meta-analyses from different sources indicate that metformin benefit GDM more than insulin in terms of less maternal weight gain and neonatal hypoglycaemia. Thus, it is reasonable to include metformin as first-line agent in GDM management and advice every GDM to receive metformin. However, there is increased risk of failure rate and prematurity with metformin therapy. This leads us to answer the question: how should metformin be given to benefit GDM the most?

In our study, over half of metformin-treated GDM women (55.8%) were required to add appropriate insulin for optimal glycaemic control. The rates reported by previous studies of metformin in gestational diabetes range from 0 to 46.6%, which is lower than our study (Chapter 2: Table 2.3). This could be due to variatons in target pre-meal and post-meal glucose levels. Tertti and his group have aimed for similar pre and post meal glucose targets(<5.5 mmol/L and <7.8 mmol/L, respectively) as our study where the failure rate was found to be 18% which is 3 times less than the failure rate in our study. This might be due to differences in diagnostic glucose values where they used fasting and 2hr postprandial values of less than 4.8 and 8.7 mmol/L respectively. Thus, they might have much more patients started on metformin therapy, but with similar

target as our study and thus, this might possibly have affected the failure rates. Moreover, the failure rate in our study was almost doubled than the study done by Corbould group (36%) (Corbould *et al* 2013). They used extended-release metformin, whereas our GDM women received normal metformin. An RCT done by Ijas *et al* also gave metformin retard to GDM women aiming at the target fasting levels of less than 5.3 mmol/L and reported 32% failure rates (Ijas *et al* 2010). Hence, it could be assumed that the type of metformin drug should be taken into consideration if we would increase the success rate of metformin monotherapy. It has been suggest that large proportion of high-risk ethnic group can increase the percentages of failure rate (Chapter 2: Table 2.13). Almost 80% of GDM women in our study are Europeans but the failure rate was much higher than those with similar proportion of European population (Tertti *et al* 2008; Niromanesh *et al* 2012). This also meant that the greater percentage of women in our study were exposed to poor glycaemic period before insulin was added on. The pregnancy outcomes in metformin failure group were poorer than metformin alone group, e.g. higher percentages of prematurity in metformin failure arm. Yet, the outcomes were favourable than insulin alone group and even showed better glycaemic control in metformin failure group.

Our study has illustrated the characters of GDM women that could differentiate the groups of patients on metformin who are likely to add supplementary insulin. GDM women who were older at booking, who initiated metformin earlier, higher fasting glucose and glycated haemoglobin at mid-pregnancy, required additional insulin. The RCT study of Ijas and his group in metformin in GDM group have reported that higher BMI at first antenatal visit and higher fasting glucose at OGTT are associated with metformin failure group (Ijas *et al* 2010). Similarly, the prospective study with large

sample sizes (n=465) done by Goh *et al* has found that raised levels of fasting and postprandial glucose at OGTT, higher BMI and GDM women with history of chronic hypertension are more likely to need supplementary insulin (Goh *et al* 2011). The causal factors involved in metformin failure among type 2 diabetes are extensively studied by Brown and his group and they have suggested that initiating metformin soon after diagnosis can preserve beta cell function and along prolong the effectiveness of metformin (Brown *et al* 2006). Historically, metformin is effective for decreasing fasting hyperglycaemia because of its action on reduction of hepatic glucose output. The high rates of metformin failure among GDM with higher fasting group in our study could be due to the slower onset of action of metformin. If metformin is initiated early in pregnancy, there may be lesser failure rate. Thus, if the treatment strategy of metformin is developed based on the maternal characters at the time of diagnosis, it can ensure consistent proper glycaemic control by addition of appropriate insulin to those with high chance of treatment failure.

We noted that the three most significant predictors of metformin failure were fasting glucose at OGTT, glycated haemoglobin at OGTT and age at booking. These three indicators are clinically useful because the information are easily applicable for healthcare providers who are keen to initiate metformin. With this criteria, GDM with high risk of metformin failure are easily identified and can be better prepared for insulin use. One small study reported that if fasting glucose was ≤5.2 mmol/l at OGTT, 93% of GDM could be controlled on metformin alone where their target fasting glucose was ≤5 mmol/l[71]. Likewise, we found that fasting glucose at OGTT for metformin failure >4.8 mmol/L detected with 69% sensitivity and 62% specificity. Moreover, we also reported stand-alone criteria with HbA1c at OGTT (>5.5%) and

age (≥30 years) which could be the best alternative for fasting glucose at OGTT at its convenience. We also established the best criteria for metformin failure which could provide the maximum sensitivity over metformin failure with minimal effect on specificity. The criteria were as follows: 1) GDM whose fasting glucose was ≥4.8 mmol/l at OGTT or 2) if fasting glucose was<4.8 mmol/l but gestational age at dietary failure was $\leq 27^{+5}$weeks. With this criteria, the sensitivity and specificity for metformin failure in GDM were 87% and 64%, respectively. We believe that these indicators have practical benefit as it is based on readily available and uncomplicated information to health care professionals who are keen to start metformin. This protocol can enable healthcare professionals at primary centre to treat GDM more confidently and can also reduce the numbers of GDM referred to tertiary centre for insulin treatment. Moreover, this may also allow reallocation of budgets dedicated for insulin care to other important areas such as dietary and lifestyle education for these GDM women.

In the near future, the prevalence of GDM will be tripled if IADPSG criteria is accepted widely (Liao *et al* 2014). We found that if those GDM identified by new IADPSG fasting glucose criterion which is ≥5.1mmol/l are given metformin, almost 80% of them are like to develop metformin failure. Likewise, the new 2015 NICE criterion (fasting ≥5.6mmol/L) which have identified similar prevalence of GDM as IADPSG and found the same positive predictive power for failure rate with metformin. This implies that for these newly identified GDM, increased risk of metformin failure could be expected. Being a convenient and better alternative to insulin, the use of metformin should not be restricted; but rather, for these particular GDM with high risk of failure, we should carefully monitor their glycaemic levels and educate them early

about the risk of metformin failure. Alternatively, addition of insulin to those with high chance of metformin failure could avoid unnecessary risky titration period.

Rowan and her group have found the increased risk of prematurity metformin treatment than insulin with the odds of 1.60 (95% confidence interval: 1.02 to 2.52), although they did not state the reason behind. We found that increased risk of prematurity among metformin failure than metformin alone groups (20.3% vs 8.9%). It could be possibly related to 1) gastrointestinal side-effects of metformin which mimic labour pain leading to delivery or 2) longer duration of suboptimal glycaemic control during escalation of metformin, thereby, promoting pre-eclampsia or similar adverse pregnancy outcomes necessitating immediate labour or 3) alteration of calcium metabolism in the gut, resulting in disturbance in uterine contractility (Rowan *et al* 2010; Bauman *et al* 200; Durnwald *et al* 2011). Previously, metformin failure in GDM population was studied by Corbould *et al* group where they did not find any relationship between metformin non-responders/failure groups and prematurity (Corbould *et al* 2013). The probable reason for differences could be that all the metformin failed GDM women in Corbould group study were switched to insulin treatment and they defined this group as metformin non-responders, whereas the failure group in our study are those with addition of insulin on top of metformin. This finding was controversial as this study included only 25 metformin users and may not have enough sufficient statistical power. Moreover, they did not report glycemic control after treatment. We also have found similar glycated haemoglobin (HbA1c) levels between these two groups and thus the prematurity rate was higher in metformin failure group. In Goh 2011 study where insulin was initiated at the introduction of metformin, the prematurity rate of metformin was similar to di*et al*one group and also

lower than insulin alone group (Goh *et al* 2011). Moreover, the prematurity in the metformin failure group was comparable to those in metformin monotherapy group. Thus, it is hoped that if certain GDM with potential high risk of requiring insulin addition earlier in pregnancy, it might possibly reduce the associated prematurity risk of metformin therapy in GDM. However, the confidence interval of prematurity in our study was very wide and thus, we would suggest that the association between metformin failure group and prematurity risk and the possible risks imposed should be further examined by a future prospective study.

We found that the average glycaemic control was better in metformin failure GDM women than in insulin therapy alone. As metformin failure women were diagnosed earlier, this might probably due to the fact that women with metformin plus supplementary insulin were on intensive glycaemic treatment earlier. Yet, the effect was found to be significant after adjusting gestational age at OGTT. Thus, another possibility could be that women on metformin will have more stable day-to-day glycaemic levels than insulin alone group. Regarding risk of prematurity risk, metformin failure group were almost 3 times more likely to deliver premature babies than insulin monotherapy group, although the effect did not reach the significant level. Thus, the costs and benefits to offer metformin to those with potential risk of metformin failure are still controversial.

Strengths and Limitations

As all the available eligible GDM women with diverse ethnicity were included, the criteria can be widely applicable. Moreover, we applied insulin addition strategy to the metformin failure GDM population, which are adapted intervention strategies in

several countries. This might allow easy application to clinical practice. However, our studies have a number of limitations. Firstly, the protocol for metformin failure was developed from retrospective cohort data and thus, a prospective study using our criteria to validate our findings are required. Secondly, despite our effort to exclude history of pre-gestational diabetes in the study, our population might have mixed with hidden pre-gestational diabetes whose signs and symptoms are undetected early in pregnancy. This should be considered in future prospective studies. Furthermore, because of the nature of research questions, we did exploratory analysis rather than hypothesis testing. Moreover, although we found possible association of metformin failure and prematurity, our study may not have enough power to detect differences in other important pregnancy outcomes. Thus, a well-designed prospective or randomized study with adequate sample size should be conducted to confirm our results before implementing these findings into practice. In addition, we would advise the following duplicate studies to include important outcomes of metformin like maternal weight gain and lipid profile.

In conclusion, there is high chance of metformin failure rate. Early initiation of metformin at diagnosis of GDM would allow more GDM with metformin monotherapy considering its slower onset. The early detection of those GDM with high probability of metformin failure would allow physicians to enable individualized treatment strategy but also help better prepare the mothers for insulin initiation. Finally, the healthcare resources to GDM could be better reallocated if the number of GDM women with insulin are reduced.

CHAPTER 5

MATERNAL VITAMIN B12, FOLATE AND HOMOCYSTEINE AS DETERMINANTS OF GLYCEMIA AND BIRTHWEIGHT IN GDM

(STUDY 3)

5.1 Introduction

The adequate supply of micronutrients are very important in pregnancy for foetal growth and development. Being major regulators of methionine:homocysteine (one carbon transfer) cycle, the imbalance or inadequacy of vitamin B12 and folate is important for DNA synthesis and repair. During pregnancy, folate insufficiency is usually responsible for increased homocysteine levels rather than vitamin B12 deficiency (Yajnik *et al* 2005). Nevertheless, increased homocysteine levels due to any cause in pregnancy are related to neural tube defects, small-for-gestational age(SGA)(Hogeveen *et al* 2012), low-birth-weight(LBW)(Sukla *et al* 2013), pre-term delivery (Knudtson *et al* 2004) and pre-eclampsia (Kahramaner *et al* 2013).

Because of short-term storage nature of folates in the body and the increasing concerns of folate-deficiency induced hyperhomocysteinaemia-related adverse pregnancy outcomes like neural tube defects, periconceptional folic acid (400ug-5mg) is recommended to every pregnant woman in their early months in many countries. In UK, women with planned pregnancy are encouraged to take folic acid 400ug/day which is much higher than the dosage recommended for normal adult women (100ug/day) before conception and throughout pregnancy. The higher folic acid dosage of 5mg is given to those who are high risks such as previous history of neural tube defects and diabetes in developed countries like UK. Whereas, in developing countries like India, 5mg folic acid is recommended to every pregnant woman owing to high prevalence of nutritional deficiencies. Regarding vitamin B12 intake during pregnancy, it is not recommended usually because of large pool of vitamin B12 storage in liver, but vitamin B12 deficiency is quite common during pregnancy due to increased demand (Bawazeer 2011). This was seen in almost half of UK pregnant

women despite non-restricted diet, probably related to increased intakes of frozen and processed meat (Adaikalakoteswari *et al* 2015). Similarly, one Indian study with majority of vegetarian population has also reported high prevalence of vitamin B12 deficiency in the presence of high folate levels during pregnancy (Krishnaveni *et al* 2009).

Therefore, there is much likelihood to have seen vitamin B12 deficiency at the high levels of serum folates which might possibly change the physiological function during pregnancy. There are also some data to support that high intakes or serum levels of folate in vitamin B12 deficient state are associated with increased cardiovascular risks, marked by hyperhomocystinemia (Selhub *et al* 2007), insulin resistance (Yajnik *et al* 2008) and obesity (Adaikalakoteswari *et al* 2014). Similarly, maternal high folate and low vitamin B12 levels can increase low birth weight (LBW) (Gadgil *et al* 2014), small-for-gestational-age(SGA) (Dwarkanath *et al* 2013), adiposity and insulin resistance (Yajnik *et al* 2008). The possible mechanism of low vitamin B12-high folate effects was postulated by Yajnik *et al* group that folic acid could not work at full potential during vitamin B12 deficiency due to folate trap (also defined as functional folate deficiency). At the same time, a meta-analysis of folate supplementation to type 2 diabetes has reported to be associated with reduction of average HbA1c by lowering homocysteine levels (Sudchada *et al* 2012) which can possibly reduce cardiovascular risks. On the other hand, high folate intakes during pregnancy can increase risk of SGA in vitamin B12 deficient setting which may be due to be poor placental blood flow (Dwarkanath *et al* 2013). Thus, it is likely for folic acid to have benefit on metabolic outcomes, it is important to have adequate vitamin B12 levels.

Accordingly, to understand the role of micronutrients on both glycaemia and pregnancy outcomes, gestational diabetes mellitus (GDM) is an useful population to study. Folic acid supplementation and opportunistic fortification of folic acid is common in UK. This may be the reason for low levels of folate deficiency in UK (Adaikalakoteswari *et al* 2014). However, vitamin B12 deficiency is also common. Therefore, this study aims to understand the association between serum folate levels with plasma glucose levels and birth weight in normal and insufficient B12 GDM women.

5.2 Methods

5.2.1 Study population and data collection

The eligibility criteria for date and location setting of this study was described in chapter 4. All GDM women with available vitamin B12 and folate levels were included. If the woman had more than one pregnancy during the period, each pregnancy was considered as a separate case. Multiple pregnancies, still-birth pregnancies and pregnancies transferred to other hospital were excluded. The diagnosis and management of the included GDM women were also described in Chapter 3.

In our centre, all the pregnancies were given folic acid (400ug/day) at the time of booking and is continued until delivery. Although it is reported that vitamin B12 deficiency (<150 pmol/L) is not uncommon in UK pregnant population, it is not supplemented routinely yet. Similarly, the measurements of serum vitamin B12 and folate levels were not included in antenatal care. In this hospital, one consultant mainly in charge of GDM clinic regularly requested serum vitamin B12 and folate measurement routinely because of the awareness of high prevalence of vitamin B12 deficiency in west midlands pregnancy since 2009. If the women was found to be vitamin B12 deficient (<150 pmol/L), IM B12 injection of 1000 IU was given successively and repeated as required. All available data of vitamin B12, folate and homocysteine of these GDM women were collected from the hospital electronic records and if the levels were measured more than one time during the same pregnancy, the date closest to the OGTT date was included. Plasma vitamin B12 and folate levels were determined by electrochemiluminescence immunoassay and binding

assay (Elecsys and cobase immunoassay analysers, Roche Diagnostics, USA) respectively and plasma total homocysteine by automated High Performance Liquid Chromatography (HPLC) method.

The following maternal and neonatal variables were included: maternal vitamin B12, folate and homocysteine levels, folic acid supplement dose and date, vitamin B12 injection date, gestational age at blood withdrawal, maternal age, height, weight, ethnicity, parity, gestational age at OGTT, fasting and postprandial levels at OGTT and postpartum, HbA1c throughout pregnancy, type of medication, smoking status, birth weight, gender and gestational age at delivery. Vitamin B12 deficiency was defined as above; low folate as <9 nmol/L and high homocysteine as ≥14umol/L. The ratio of folate:vitamin B12 was also done by dividing folate by vitamin B12 levels, then multiplied by 1000. The GROW customized weight centiles was used to determine the centiles of newborns and small for gestational age (SGA) and large for gestational age (LGA) were defined by < 10% centiles and >90% centiles, respectively (Clausson *et al* 2001; Mikolajczyk *et al* 2011). This growth chart was adjusted for maternal height, weight, ethnicity, parity, gender and gestational age at delivery. There is strong evidence that GROW customised growth charts were better predicted models for perinatal morbidity and neonatal arthropometric measures (Gardosi *et al* 2004). Moreover, SGA identified by individually adjusted growth centiles were better representatives of IUGR (Clausson *et al* 2001).

5.2.2 Sample size calculation

A-priori statistical power analysis was performed using a software available online[231]. The anticipated effect size used for this calculator was (f^2) 0.02 for small, 0.15 for medium and 0.35 for large. A power analysis for multiple linear regression with 4 predictors (folate:vitamin B12 ratio, age, BMI, parity) with 80% power and significance levels of 0.05 required a sample size of 84 GDM to detect f^2 of 0.15 in normal vitamin B12 subgroup.

5.2.3 Statistical analysis

Statistical analysis were performed using SPSS programme version 21.0 (SPSS Inc., Chicago, IL, USA). Complete case analysis (serum vitamin B12, serum folate, glucose levels at OGTT, birth weight, age, BMI, parity) was performed. The skewed data were log-transformed and presented as mean and standard deviation. Among all continuous data, only birth weight was normally distributed. The log-transformed data were used in further analysis. Continuous data were described in mean±standard deviation (SD) and categorical data were described in number(n) and percentages(%). P value of 0.05 was considered as statistically significant. Scatter-plot was used to reveal the association between maternal micronutrient levels and glucose levels among normal and deficient vitamin B12 groups.

5.2.3.1 Question being addressed:

1. What is the association between serum vitamin B12 and serum folate at mid-pregnancy with BMI, serum homocysteine levels, plasma glucose levels (fasting and 2hr postprandial) at mid-pregnancy and 6 weeks post-partum and their babies' birth weight or centiles in GDM women?

Statistical model applied: Pearson’s correlation

2. Is there any difference in the association of serum folate levels at mid-pregnancy with above mentioned dependent variables in question (1) between low and normal vitamin B12 groups?

Three different analytical strategies using appropriate statistical models applied:

i) Vitamin B12 grouping – low (<150pmol/L) and normal (≥150pmol/L) and Pearson’s correlation model

ii) Serum folate levels divided by serum vitamin B12, then multiplied by 1000 and Pearson's correlation model

3. Does the association between serum folate levels at mid-pregnancy with plasma glucose levels change after controlling for age, BMI and parity (and homocysteine)?

Statistical model applied: Multiple regression model

Analytical strategy:

Outcome variable: Plasma glucose (fasting and postprandial) at mid-pregnancy and 6-weeks post-partum

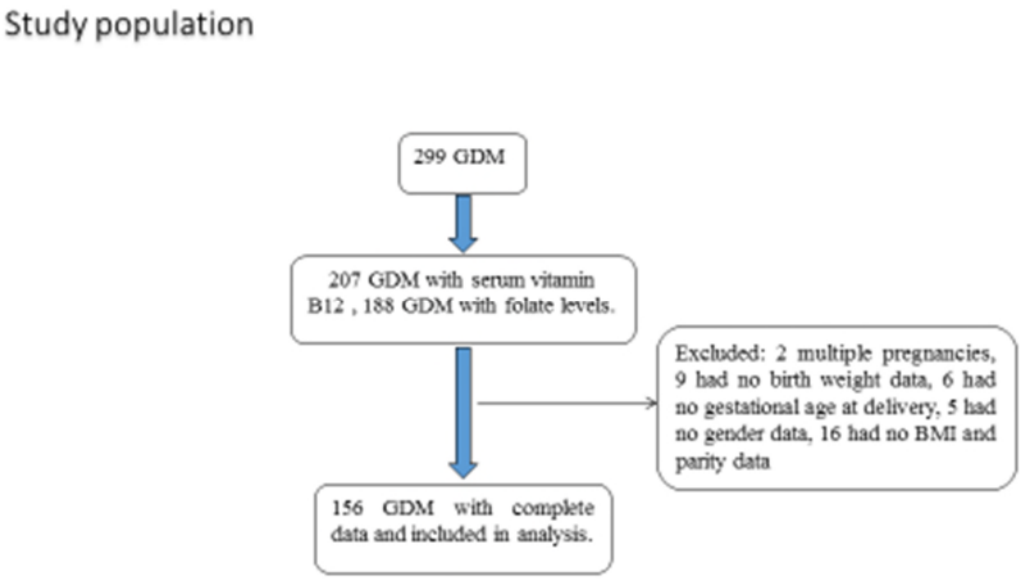
Step 1: Serum folate levels

Step 2: Age, BMI, Parity and Serum folate levels

Step 3: Age, BMI, Parity, Homocysteine and Serum folate levels

5.3.Results

Figure 5.1 Overview of selection of GDM women included



Out of 299 eligible GDM women, 207 women and 188 women had recorded data for serum vitamin B12 and folate levels respectively. All the women who had serum folate recorded had been measured together with serum vitamin B12. After exclusion of GDM with no folate data, complete data were examined fasting and 2 hour glucose at OGTT, parity, BMI, age, birth weight, gender and gestational age at delivery in 188 GDM women. Complete data for analysis were available for 156 GDM and included (Figure 5.1)

5.3.1 Demographic characteristics of GDM mothers and babies

The characters of mothers and babies including blood indices were presented in Table 5.1. Data on folic acid supplementation were available from 155 GDM. Majority (85.8%) women had taken folic acid 400ug antenatally and 2 GDM took folic acid other than 400ug. Out of 156, 80% of women lived in own or rent accommodation. The prevalences of B12 and folate deficiency are 32.7% and 5.1% respectively at median gestational age of 26^{+6} weeks. weeks(interquartile range(IQR)26^{+0}, 29^{+2} weeks). If low and high folate groups were defined The prevalences of low and high folate levels defined by <3.8ug/L and >16ug/L were 3.8% and 17.3% respectively. There is only one GDM with high homocysteine (≥14umol/L). Over fourth-fifths (85.3%) of GDM women were Europeans. The incidences of LBW and SGA are 7.1% and 14.1% respectively. Two-thirds of the women were on metformin and over half of them needed additional insulin.

Table 5.1 Characteristics of GDM women

n=156	Median (IQR)	Mean±SD
Age (years)	31.0 (27.0,35.8)	31.09±5.6
Ethnicity (n=154) i)South Asians ii)European iii)Others	 10(6.4%) 133(85.3%) 11(7.1%)	
Smoking	19(12.2%)	
Parity Nullip Multip(>/=2)	 60(38.5%) 38(24.4%)	
Weight (kg)	82(70,99)	84.4±19.3
Height (cm)	163(159,168)	162.9±7.3
BMI	30.9(27.3,36.1)	31.8±6.8
Pregnancy-induced hypertension(PIH)	6(3.8%)	
Treatment type (n=134) i)Diet ii)Diet+Metformin iii)Diet+Metformin+Insulin iv)Diet+Insulin	 13(8.3%) 39(25.0%) 59(37.8%) 23(14.7%)	
Vitamin B12 (pmol/L) B12 deficiency(<150 pmol/L)	223.0(184.3,290.5) 51 (32.7%)	247.1±91.9
Folate (ug/L) Folate deficiency(<9 nmol/L)	9.9(6.6,14.0) 8 (5.1%)	10.5±4.7
(Folate:B12) x 1000	41.1(29.2,56.9)	45.1±21.5
Homocysteine(umol/L)(n=83)	5.0(4.0,7.0)	5.9±2.5
OGTT i) Fasting (mmol/L) ii) 2hr post prandial (mmol/L)	 4.9(4.5,5.6) 8.6(8.0,9.5)	 5.1±0.9 8.8±1.5
Post-partum(n=62) i) Fasting (mmol/L) ii) 2hr post prandial (mmol/L)	 4.9(4.6,5.3) 5.4(4.6,6.7)	 5.0±0.7 5.8±1.9
Gestational age (GA) at Delivery (weeks)	38+2(38+0,38+5)	38+2±0+7
Birthweight (grams) Low Birth weight(<2500g) Macrosomia(≥4000g)	3200(2958.8, 3557.5) 11 (7.3%) 6(3.8%)	3217.1±455.5
Centiles Small-for-gestational age(SGA) Large-for-gestational age(LGA)	35.4(16.5,71.3) 22(14.1) 12(7.7)	43.5±30.3

5.3.2. Relation between serum folate, vitamin B12, their ratio and glucose levels

There was a significant association between serum glucose levels at diagnostic and postpartum GTT with folate levels, but not with vitamin B12 levels (Table 5.2). Among vitamin B12 subgroups, folates were inversely related to fasting glucose levels at OGTT and postpartum GTT in normal vitamin B12 GDM subgroup only (Figure 6.2 and Figure 6.3) whereas average HbA1c levels were correlated with folates in normal and deficient B12 GDM. The association was significant after adjusting the confounders such as age, BMI and parity (Table 5.3) and the R^2 change was increased with inclusion of homocysteine in the model (Table 5.3). Among normal vitamin B12 GDM subgroup, the ratio of folate:vitamin B12 was associated significantly with fasting glucose at mid-pregnancy and postpartum GTT (Table 5.2). Homocysteine levels were strongly correlated with folate levels but not with vitamin B12 while maternal BMI are negatively and significantly related with both serum vitamin B12 and folate levels. <3.8ug/L and >16ug/L. Serum B12 and folate are highly correlated ($r=0.341$, $p<0.001$) but only folate levels are negatively associated with homocysteine($r= -0.46$, $p<0.001$, $n=83$). There was no difference in vitamin B12, folate and homocysteine levels among diet, metformin, metformin+insulin and insulin treated GDM (data described in Chapter 3).

Table 5.2: Pearson's correlation-plasma glucose, homocysteine, BMI and birth weight correlates with serum micronutrients levels

	All GDM				Normal B12GDM				B12 insufficient GDM	
	Vitamin B12		Folates		Folates		(Folates:Vitamin B12)*1000		mid-pregnancy (n=51)	Postpartum (n=19)
	mid-pregnancy (n=156)	Postpartum (n=62)	mid-pregnancy (n=156)	Postpartum (n=62)	mid-pregnancy (n=105)	Postpartum (n=43)	mid-pregnancy (n=105)	Postpartum (n=43)		
fasting	-0.10(NS)	0.09(NS)	-0.19(<0.05)	-0.27(<0.05)	-0.21(<0.05)	-0.36(<0.05)	-0.18(NS)	-0.30(<0.05)	-0.06(NS)	-0.16(NS)
2hr- PP	-0.003(NS)	-0.02(NS)	-0.14(NS)	-0.15(NS)	-0.20(<0.05)	-0.20(NS)	-0.18(NS)	-0.10(NS)	-0.01(NS)	0.11 (NS)
Average HbA1c	-0.069(NS)						-0.21(<0.05)			
tHcy	-0.04(NS)		-0.46(<0.001)				-0.43(<0.001)			
BMI	-0.29(<0.01)		-0.22(<0.001)		-0.25(<0.01)		-0.03(NS)		-0.07(NS)	
Birthweight	-0.02(NS)				0.06(NS)		0.05(NS)		0.01(NS)	
Centiles	0.09(NS)				0.15(NS)		0.05(NS)		0.03(NS)	
NS= Not Significant										

Figure 5.2 Scatterplots of Relationship between Folates and Fasting glucose at mid-pregnancy OGTT

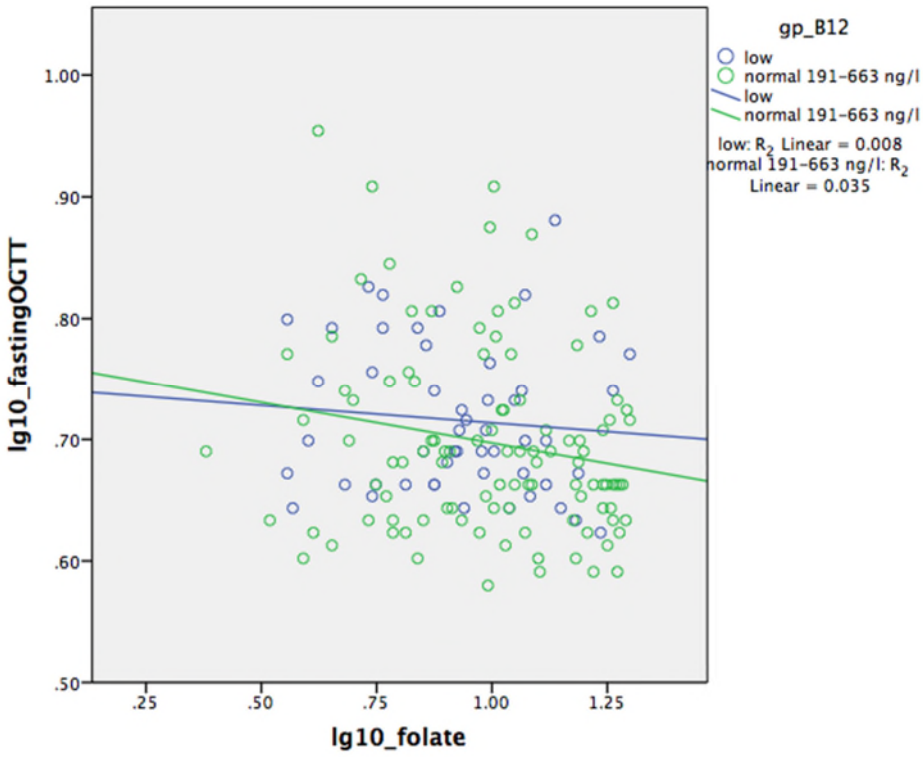


Figure 5.3 Scatterplots of Relationship between Folates and Fasting glucose at post-partum GTT

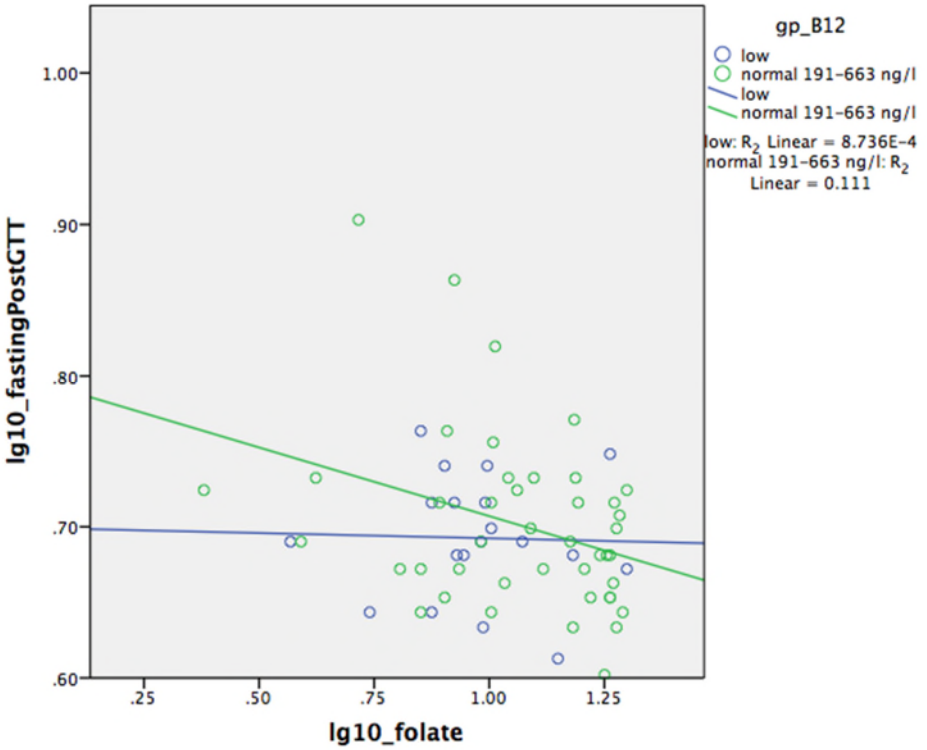


Table 5.3: Multiple regression for fasting glucose at diagnostic and postpartum glucose tolerance test

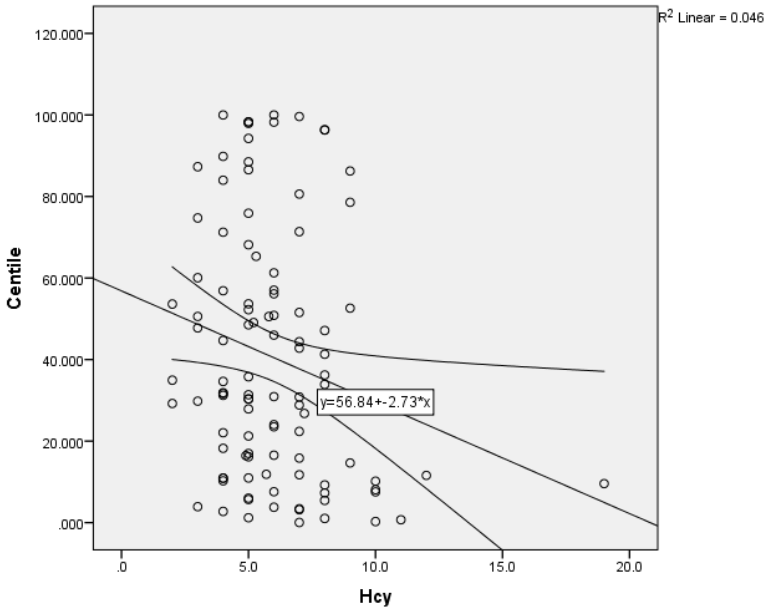
	All GDM		Normal B12GDM		B12 insufficient GDM	
folate	mid-pregnancy (n=156)	Postpartum (n=62)	mid-pregnancy (n=105)	Postpartum (n=43)	mid-pregnancy (n=51)	Postpartum (n=19)
β	-0.19(<0.05)	-0.27(<0.05)	-0.21(<0.05)	-0.36(<0.05)	-0.06(NS)	-0.16(NS)
Adjusted β (Age, BMI, Parity)	-0.17(<0.05)	-0.32(<0.05)	-0.18(<0.05)	0.38(<0.05)	-0.06(NS)	0.21(NS)
	n=83	n=37	n=52	n=23	n=31	n=14
β	-0.24(<0.05)	-0.31(<0.05)	-0.32(<0.05)	-0.32(NS)	0.02(NS)	-0.24(NS)
Adjusted β (Age, BMI, Parity)	-0.21(=0.09)	-0.40(<0.05)	-0.30(<0.05)	-0.42(NS)	0.12(NS)	-0.05(NS)
Adjusted β (Age, BMI, Parity, homocysteine)	-0.30(<0.05)	-0.36(<0.05)	-0.44(<0.01)	0.31(NS)	0.12(NS)	-0.06(NS)
NS= Not Significant						

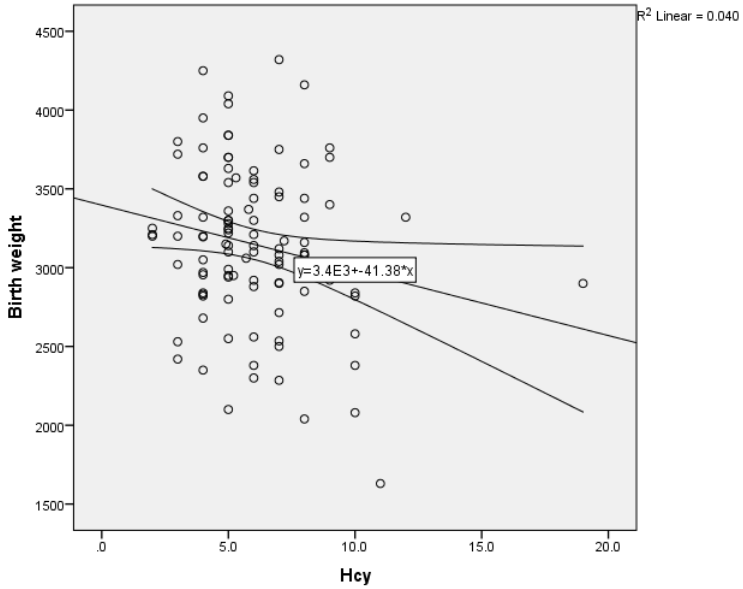
5.3.3. Relation between serum folate, vitamin B12, their ratio and birth weight

There was no association between serum folate, vitamin B12 and their ratio with birth weight or centiles (Table 5.2) (Figure 5.4). There was no relationship of these micronutrient levels with either SGA or LBW. However, there was a significant negative linear correlation between maternal total homocysteine and centiles and birth weight ($r=-0.22$, $p<0.05$ and $r=-0.10$, $p=0.39$). In multiple regression models, it was found that homocysteine, age and smoking were major contributors of centiles and after adjusting age and smoking, 1SD rise in homocysteine (2.5umol/L) can reduce 0.19% centiles (95% confidence interval (CI): -5.0, 0.32). Moreover, the odds ratio of 1.38 (95%CI: 1.06, 1.79) for SGA were greater than 1, indicating that for every one umol/L increase in maternal homocysteine levels, it was 1.38 times likely to increase SGA risk, controlling for age and smoking. The mean birth weight and centiles of vitamin B12 deficient group were 64 g higher than normal B12 GDM (3260.4±487.0g vs 3196.1±440.0g, $p=0.41$) (Figure 5.5).

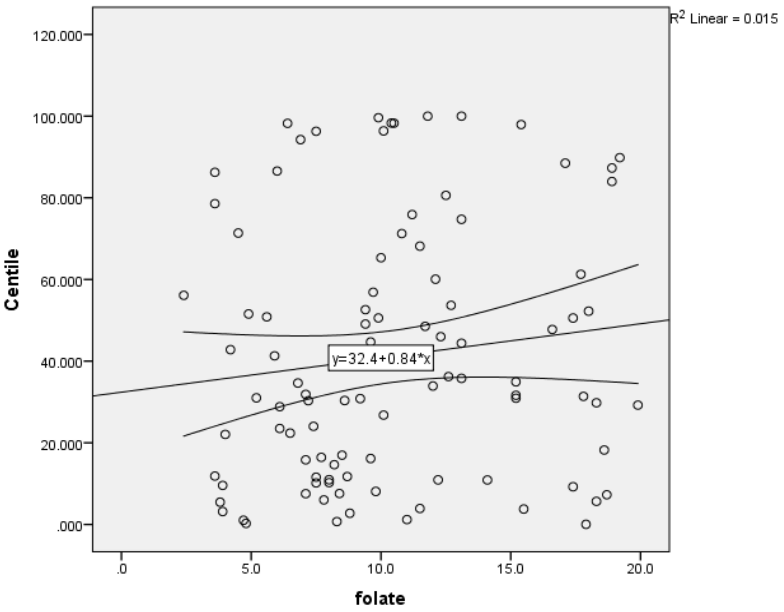
Figure 5.4: Scatter-plots of homocysteine, vitamin B12 and folate vs centiles and birth weight. (The lines represent mean±standard deviation.)

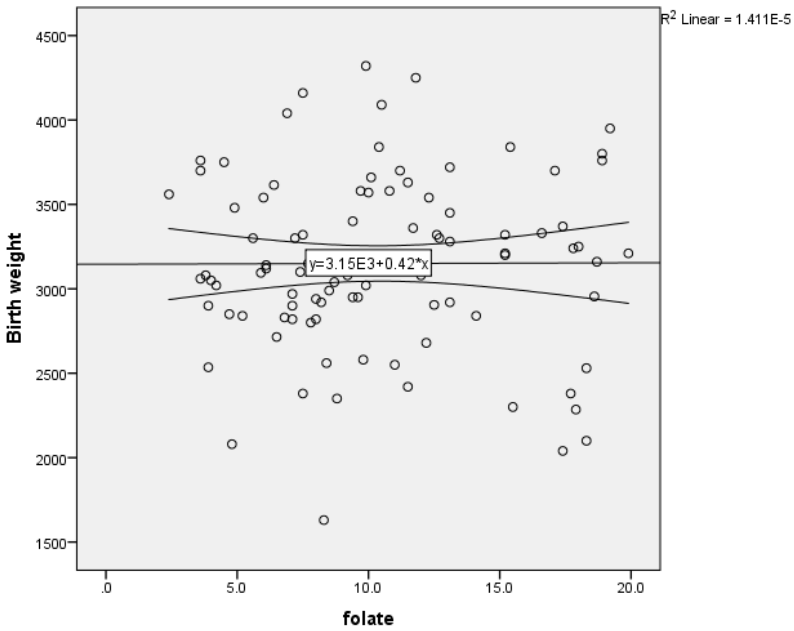
A) Plasma Homocysteine levels vs Centiles and Birthweight



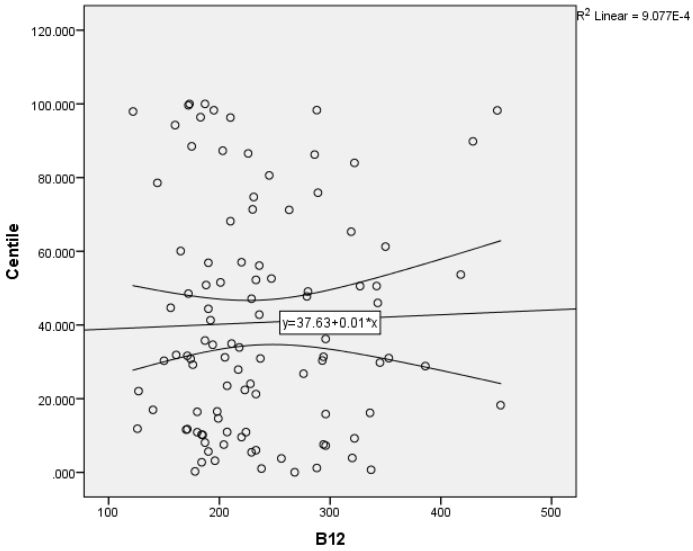


B) Plasma Folate Levels vs Centiles and Birthweight





C) Plasma Vitamin B12 Levels vs Centiles and Birthweight



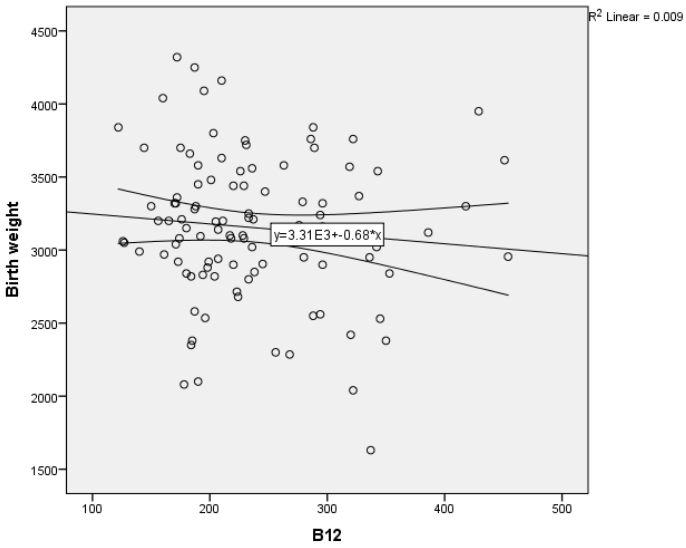
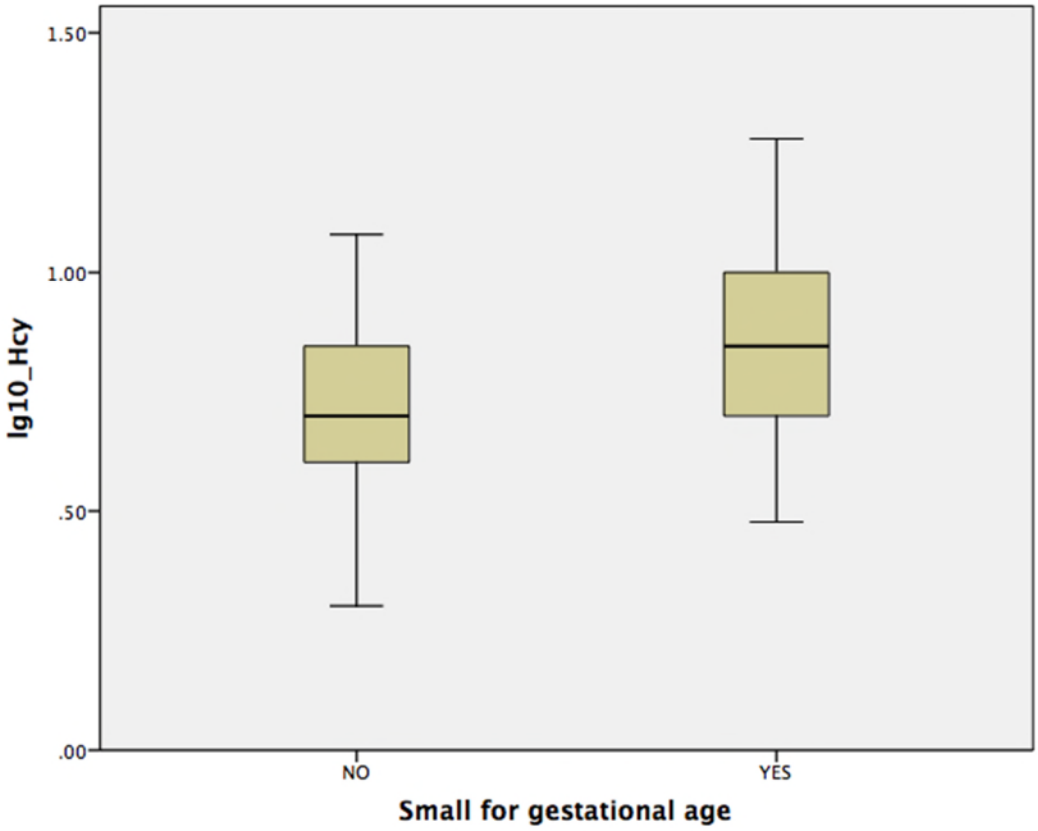


Figure 5.5: Differences in mean total homocysteine levels between Appropriate for Gestational Age (AGA) and Small for Gestational Age(SGA)



5.4 Discussion

The awareness of neural tube defect due to folate deficiency has led to give 400 ug folic acid supplements to all pregnant women in UK, ideally before conception and throughout pregnancy. Although the majority of the pregnancy are not well-planned, the health-conscious population in general are likely to take folic acid and thus the women at booking visit have usually on folic acid supplements. However, this is not accompanied by increase in the intake of other micronutrients especially vitamin B12, deficiency of which can result in imbalance of methionine-homocysteine cycle because of folate trap. Consequently, the increased homocysteine levels can impair vascular blood flow resulting in higher incidence cardiovascular and peripheral vascular diseases and adverse pregnancy outcomes due to placental vasculopathy including SGA (Hogeveen *et al* 2012; Humphrey *et al* 2008; Khandanpour *et al* 2009). In fact, vitamin B12 deficiency in pregnancy is associated with higher risk of GDM (Krishnaveni *et al* 2009). Thus, this study aimed to examine the relationship of serum folate and vitamin B12 levels during pregnancy with other metabolic risk markers such as homocysteine, glycaemia and SGA and the importance of their imbalance. We have found that serum folate levels were inversely associated with serum glucose levels in GDM with normal vitamin B12, but not B12 deficient. This relationship was also found to be homocysteine-independent. Among vitamin B12 deficient group, there is no association between folate and glucose denoted by folate:vitamin B12 ratio. We have performed this analysis among vitamin B12 deficient GDM subgroup because the interest was lowering of serum glucose levels by folate in the presence of vitamin B12 deficiency. Moreover, in these 400ug folate supplemented GDM, serum folate did not influence on the birth weight of babies and their centiles. Thirdly, it was found that

vitamin B12 deficiency in GDM was significantly associated with increased BMI. This might suggests that despite being in normal range, lowering homocysteine levels could reduce risk of SGA. As found in type 2 diabetes population, the increased homocysteine levels in this GDM population were strongly correlated with folate and not vitamin B12 levels. This further strengthens the argument that folate is the strong driver of homocysteine levels in vitamin B12 sufficient population.

5.4.1 Micronutrients and glycaemia

Although studies found that high folic acid levels in low vitamin B12 status are associated with increased risk of insulin resistance (Krishnaveni *et al* 2009, Yajnik *et al* 2008)), the current study did not show this as all GDM included received 400ug folic acid compared to 5 mg folic acid in other studies. We have also found that lowering glycaemic levels by folate in normal vitamin B12 GDM. This finding was in consistent with systematic review on glucose lowering effects of folic acid in type 2 diabetes although the systematic review did not include vitamin B12 level (Sudchada *et al* 2012). This study has also reported that this effect was due to lowering of homocysteine levels. Likewise, one Indian study in pregnant women has reported the association of high folate and low vitamin B12 imbalance with highest increased homocysteine levels (Gadgil *et al* 2014). However, in our current study, this relationship was found to be homocysteine-independent. This might suggest the effect of folate on glycaemia rather than homocysteine-mediated mechanism. There is also evidence that serum folate levels facilitate endothelial function by directly interacting with endothelial nitric oxide synthase in type 2 diabetes (Steware te al 2011).Accordingly, this indicates that the effect of folate on glycaemia could be rather direct, i.e, folate effects on endothelium rather than homocysteine-induced endothelial

oxidative stress. Similarly, Selhub *et al* have reported that serum folate levels are inversely associated with serum methylmalonic acid and homocysteine levels generally, but if folate levels >20nmol/L in low B12 levels, the relationship is found to be reverse (Selhub *et al* 2007). Thus, we concluded that for folates to have positive effects on glucose levels, it is essential to have normal vitamin B12 levels in GDM.

In this study, we have given B12 injection of 1000 IU to deficient GDM soon after lab results and have found that serum vitamin B12 levels return back to normal within a week. Despite this, the correlation of serum folate and glucose levels at postpartum GTT did not improve in those with formerly vitamin B12 deficient group. This might suggest the physiological importance of vitamin B12 for cell function rather than mechanical role. Recently, the study of vitamin B12 in adipocyte cell culture has also reported that vitamin B12 deficiency reduced methylation potential and increased the gene expression of cholesterol biosynthesis (Adaikalakoteswari *et al* 2015). This also indicates that vitamin B12 has a priming effect on cells, i.e, needs to enter and become incorporated into cell functional structure, in order for folate to have an effect on lowering glucose. Moreover, it is better off using serum methylmalanoic acid levels which is more sensitive and specific for intracellular vitamin B12 deficiency together with serum vitamin B12 levels in follow-up replicated studies.

Although vitamin B12 status could modify the effects of folate on glucose in GDM, there was no direct correlation of vitamin B12 and glucose levels. But, we did see the significant negative association of vitamin B12 or folate on maternal BMI. These findings are in line with previous indian pregnancy study where reduced HOMA-IR and GDM risks were found across higher B12 tertiles at 5 year follow-up (Yajnik *et al* 2008) although they did not find any association with folate levels. Moreover, there is

evidence that maternal vitamin B12 deficiency can increase the risks of neonatal adiposity, HOMA-IR and dyslipidaemia (Adaikalakotewari *et al* 2015; Yajnik *et al* 2008; Stewart *et al* 2011). These findings might support the generational effects of vitamin B12 that it takes considerable amount of time interval for vitamin B12 to have a visible impact on metabolic function.

5.4.2 Micronutrients and Birth weight

At the same time, it has been reported that either low vitamin B12 or folate levels are associated with increased risk of SGA or low birth weight (LBW) (Christian *et al* 2003; Relton *et al* 2005; Dwarkanath *et al* 2013). Similar report was also done by Gadgil *et al* group with imbalance of these 2 micronutrient levels in pregnancy with high folate (5mg) intake [143]. Likewise, Dwarkanath and the group have reported low folate low vitamin B12 intakes in 1st trimester and high folate and low vitamin B12 intakes in 2nd trimester can increase the risk of SGA (Dwarkanath *et al* 2013). On the contrary, a randomized study done by Christian *et al* in community setting has reported that antenatal folate supplementation of 400ug can moderately reduce the risk of LBW(Christian *et al* 2003). In this GDM study, we did not find any relationship between serum folate, vitamin B12 and their imbalance with SGA and LBW. In our previous study (2005-2010) (n=38), we have reported that non-significantly higher birth weight among low vitamin B12 GDM mothers. We have seen the similar pattern in this 2009-12 GDM group with much higher sample size but the association is not significant(p=0.4) (Bawazeer 2011). Our GDM population were relatively folate adequate (94.9%) with low vitamin B12 status (32.7%) in contrast to the previous 2005 GDM study with vitamin B12 and folate deficiency of 57.9% and 24.3% respectively (Bawazeer 2011) . The discrepancies in the prevalence of vitamin B12 and folate levels

are due to sampling differences and we collected all consecutive sample in this 2009 study. The incidences of SGA and LBW in our study are 15.3% and 7.3% respectively which were much higher than being reported by other GDM studies. Furthermore, there was one GDM with homocysteine levels ≥14umol/L. Despite being in normal range, higher serum homocysteine levels were significantly associated with increased risk of SGA in GDM and they were negatively correlated with serum folate levels. Therefore, it can be assumed that decreasing homocysteine levels by increased folic acid intakes might be of benefit to bring down the incidence of SGA in GDM Moreover, maternal homocysteine levels at mid-pregnancy could be used as a guide for treatment modifier for target glucose adjustment for GDM with high risk of SGA and LGA. Considering the risk of high folate in B12 deficient we would suggest that among GDM with 400ug folic acid intakes, whether there is the effect of serum folate and vitamin B12 levels on birth weight is still controversial. However, considering detrimental effects of high folate in vitamin B12 deficient setting, increased folate dose might not be a wise solution, and thus rather, we would recommend routine check of vitamin B12 status in early pregnancy and supplementation, if deficient.

5.4.3 Strengths, Limitations and Recommendations for future studies

This study shows novel evidence of a relationship of serum folates on glucose levels during pregnancy and at postpartum among subgroups of normal and deficient vitamin B12 GDM with normal homocysteine levels, although only one-third of GDM women were available for postpartum glucose data. However, this is a usual condition in clinical practice where approximately half of GDM women attend postpartum glucose appointment. Moreover, the availability of a wide range of variables with adequate sample size allowed to explore the analysis deeper to provide more robustic evidence.

Furthermore, the use of GROW centiles helped to define SGA for wider implication and reproducible replication. The nature of population being diverse and representative are also the strengths of this study. The limitations relate to the lack of dietary measures and adiposity of offspring. In addition, to validate the effects of folate and vitamin B12 on birth weight in GDM, it would be required to conduct a prospective randomized trial with vitamin B12 supplementation alongside the 400ug folic acid supplements. Finally, examination of whether lowering down of homocysteine within normal range by high folic acid dose in the presence of normal vitamin B12 are beneficial for birth outcomes is worthwhile.

In summary, the study highlights high prevalence of vitamin B12 deficiency and its importance in folate-dependent glucose reduction. It also suggests that the timing of vitamin B12 should be adequate enough to allow physiological cell transformation. Thus, in order for vitamin B12 to benefit during pregnancy, it is more appropriate to implement vitamin B12 measurement and supplementation targeting at general population.

CHAPTER 6

ROLE OF VITAMIN B12 DEFICIENCY ON LIPID METABOLISM AND METFORMIN ACTION IN LIVER

(STUDY 4)

6.1 Introduction

Metformin is a well-established anti-diabetic drug, used in diabetes and because of its role in lowering blood lipid levels, it is increasingly used for obese patients. One of its side-effects includes decrease in serum vitamin B12 levels. It has been reported that long-term metformin use in type 2 diabetes is associated with progressive decrease in blood vitamin B12 levels in dose-dependent manner (de Jager *et al* 2010). In fact, vitamin B12 deficiency among metformin users is 2 times higher than non-metformin user (Reinstatler *et al* 2012). Thus, it is not uncommon to find vitamin B12 deficiency among metformin-treated patients.

It has been reported that vitamin B12 deficiency is associated with impaired lipid metabolism. There are also a number of reports from clinical studies that there is significant association between vitamin B12 deficiency and dyslipidaemia (Adaikalakoteswari *et al* 2014; Mahalle *et al* 2013). So far, it is suggested to occur through altered methylation by the fact that adipocyte cultured in vitamin B12 deficient culture revealed increased intracellular cholesterol levels (Adaikalakotewwari *et al* 2015). However, the role of vitamin B12 deficiency in mitochondria is still unclear yet. It is stated that MMA accumulation due to vitamin B12 deficiency impedes coenzyme Q biosynthesis, which is important for mitochondrial respiratory function. Therefore, it is possible that mitochondria function might be affected in vitamin B12 function.

Metformin primarily works by activating AMPK (adenosine monophosphate-activated protein kinase) through raising AMP:ATP ratio by partially blocking mitochondria complex I respiratory chain. Zang *et al* also reported that the lipid

lowering effects of metformin occurs through AMPK activation(|Zang *et al* 2004). AMPK is a master energy regulator of the cell. Activated AMPK in liver then subsequently phosphorylates downstream enzymes important in lipid metabolism. Phosphorylation at serine 79 of ACC (Acetyl-CoA Carboxylase) by AMPK could inhibit its action on lipogenesis while favouring fatty acid oxidation(Zang *et al* 2004). In addition, AMPK also inhibits SREBP (sterol regulatory element binding protein) which is nuclear transcription factor for lipid synthesis enzymes and thereby, decreases expression of 3-hydroxy-3-methyl glutaryl coenzyme A reductase (HMGCR) and fatty acid synthase (FAS), which are rate-limiting enzymes in cholesterol and triglyceride synthesis (Li *et al* 2011). It is thus essential to have optimal mitochondrial function for full efficacy of metformin on lipid metabolism.

In order to understand vitamin B12 induced dyslipidaemia and efficacy of metformin action in vitamin12 deficient liver cells, we have chosen human hepatoma cell (HepG2) line as a model to conduct our experiment. Liver is the storage organ of vitamin B12 in the body and it also plays a major role in the regulation of blood lipid levels. Moreover, the recommended culture media of HepG2 (MEM, Invitrogen) does not contain vitamin B12. The only source of vitamin B12 in HepG2 culture media is from the supplemented foetal bovine serum which was stated to have 120 pM levels of vitamin B12(Oltean *et al* 2003). Considering the fact that these cells are replicated successively in culture media without vitamin B12 over time, it can be assumed that vitamin B12 in these cells are lower than the required amount for optimal cellular function. It could therefore be postulated that long-term in-vitro culture of HepG2 can induce vitamin B12 deficient hepatocyte model. The passage number in this experiment started from passage 4 HepG2 as to ensure the replication of experimental

hepatocytes in media lack of vitamin B12. We chose the vitamin B12 addition model rather than subtraction study as it resembles much more closely to the physiological condition.

The aim of this chapter are:

1) To examine how low vitamin B12 affects the lipid metabolism in liver cell line and elucidate the pathway involved
2) To examine whether vitamin B12 deficiency interferes with metformin action in liver cell line.

The molecular lab research techniques were used to understand the effects of metformin in vitamin B12 deficient setting by using HepG2 cell culture model.

6.2 Methods

6.2.1 Materials

Metformin (1,1-dimethylbiguanide), cyanocobalamin, 5-aza-2'-deoxycytidine (AZT), Anti-rabbit antibodies conjugated to horseradish peroxidase and dimethyl sulphoxide (DMSO) were purchased from Sigma. Phospho-AMPKα(Thr-172), total AMPK against AMPKα1 and AMPKα2, phospho-Akt (Ser-473), Akt, phospho-GSK3α/β, GSK3β, phospho-ACC(Ser-79), total-ACC, β-actin antibodies were purchased from cell signalling. Eagle's minimum essential medium (MEM) and foetal bovine serum were from Invitrogen. The custom taqman gene expression assays and housekeeping gene 18s rRNA from Applied Biosystems. The gene assays with their catalogue numbers included are: SREBF1 (Hs01088691_m1), SREBF2 (Hs01081784_m1), FAS (Hs00236330_m1) and HMGCR (Hs00168352_m1). The gene primers and polymerase chain reaction (PCR) test kits were from life technologies. Modified Eagle's Medium (MEM), foetal bovine serum, sodium pyruvate and antibiotics (penicillin and streptomycin) were from Invitrogen.

6.2.2 HepG2 purchase, culture and freezing

HepG2 cells were kindly contributed by Dr Ming Zhan Xue, who originally purchased them from American Type Culture Collection (ATCC). The cells were grown in MEM supplemented with 10% foetal bovine serum (FBS), L-Glutamine (2mM), sodium pyruvate (100mM) and penicillin-streptomycin (100 units/mL) in T-75 or T-175 flasks (corning cell culture flask surface area 75cm^2 or 175 cm^2 respectively). The frozen cells were dissolved in warm completed media and maintained as sub-confluent

monolayer at 37°C and 5% CO2. The media was changed the next morning and then it was changed every alternate day.

When they became 70% confluence, usually on 4^{th} or 5^{th} day, trypsinization and next cell passage were done. In brief, after removal of cell culture media, cells were rinsed 3 times with warm phosphate buffer saline (PBS); treated with 2-3ml or 5 ml of 0.25% warm trypsin-0.53mM ethylene-diamine-tetraacetic acid (EDTA) solution (Gibco, UK) to $75cm^2$ or $175cm^2$ flask respectively and kept at 37°C until cell layer was detached (usually 5-15 minutes). Trysin-EDTA solution was used to neutralize all trypsin inhibitors in FBS. Then, 10/(T-75) or 15/(T-175) ml of the complete growth media was added to halt the trypsin action and the cells were aspirated by pipetting gently to facilitate cell separation of any clump. After cells were separated individually, the cell mixture was put into 50ml falcon and centrifused at 1250 rpm for 5 minutes. The supernatant was then removed and the resulting cell pellet was re-suspended in warm media and splitted into new culture flasks (1:4). Alternatively, the cell pellet was dissolved in media containing 10%DMSO and 30%FBS and appropriate aliquots (1:10) were added into freezer tubes and kept in -80°C freezer for 24 hours. Finally, they were submerged in liquid nitrogen for longer storage.

6.2.3 Experimental steps

6.2.3.1 Experimental treatment of HepG2 cells with different concentration of vitamin B12

HepG2 cells were grown in MEM supplemented with 10% FBS and penicillin-streptomycin (100 units/mL). Cells were seeded at a density of 6000cells/cm^2 and treated with different concentrations of vitaminB12 (≥98% cyanocobalamin, Sigma-

Aldrich) (10 nM, 100 nM and 1000 nM) for 24 days (passaged on every 6th day for 4 passages) and incubated at 37°C in 5%CO2. The dose concentrations are approximated from the previous studies of vitamin B12 regulation of methionine synthase enzyme where 1.5uM and 3.6uM of vitamin B12 were added to HepG2 culture (Oltean *et al* 2003; Gulati *et al* 1999).The culture plates were kept in serum free medium overnight before extraction or before any treatment. A control culture plate with respective vitamin B12 concentration was kept alongside together with any treatment. Three independent experiments were conducted for each treatment.

For insulin treatment, HepG2 cells on 22nd day (70% confluence) in different vitamin B12 concentrations (0, 10, 100, 1000 nM) were treated with insulin (100nM) for 15 minutes before extraction.

For metformin treatment, metformin (2mM) was used to treat HepG2 cells on 23rd day morning 9.00am (22-days old and left in serum free medium for overnight) (70% confluence) in different vitamin B12 culture (0, 10, 100, 1000 nM) for 24hours before extraction on 24th day. The dose and duration of metformin was taken from the previous study of AMPK signalling of metformin on lipid in HepG2 cells(Zang *et al* 2004).

For the AZT treatment, these 22days-old HepG2 cells in vitamin B12 culture (0, 10, 1000 nM) were seeded again on 6 well plates (20% confluence) and treated with AZT (300nM) for 3 days and then left again in respective B12 supplemented normal media for another 2 days before extraction on day 27. The dose and duration of 5-AZT was taken from the previous adipocyte model (Adaikalakoteswari *et al* 2015). AZT is a cytosine analogue and is used in cell culture as a DNA hypo-methylation agent. It

replaces the cystosine in DNA synthesis, forms covalent bond with DNA methyltransferase and prevents the methylation process (Poirier S *et al* 2014).

6.2.3.2 Protein and RNA Extraction

At the end of each experiment, the HepG2 culture 6-well plates were washed with ice-cold PBS for 3 times to stop metabolism and 300µl/well of prepared Radio-Immuno Precipitation Assay (RIPA) buffer (Thermo Scientific, UK), containing a cocktail of protease inhibitors and phosphatase inhibitors [20 mM Tris–HCl (pH 7.5), 150 mM sodium chloride (NaCl), 1 mM EDTA, 1% NonidetP40, 0.5% deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 5 mM sodium fluoride (NaF), and 0.1mg/ml phenylmethylsulphonyl fluoride (PMSF) protease inhibitor] was used for protein extraction. QIAzol lysis reagent (QIAGEN, UK) of 500µl/well was added for RNA extraction. The plates were then placed on ice tray and incubated on the shaker for 10 min and cells were scraped with cell scraper, one for each condition separately. Before collecting into Eppendorf tubes, the cell suspensions were aspirated and well-mixed by pipetting. The RIPA cell lysates were centrifuged at 16,000 g at 4°C for 5 minutes. Once spun, the resultant protein-containing supernatant was then collected into new Eppendorf tubes. Both final protein and RNA extracts were kept at -80°C before further use.

6.2.3.3 Protein Quantification

Protein concentration in each sample was quantified using the Bio-Rad Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA). The frozen protein samples were left at room temperature until thawed and then vortexed for homogenous mixture. A 5 fold concentrated Bradford reagent was diluted with deionised water to produce

1X working concentration. A bovine serum albumin (BSA) protein standard concentration of 1 mg/ml was also prepared. By mixing appropriate volume of BSA and distilled water, five protein standards (0, 0.5, 1, 2, 4µg/µL) were yielded where the dye reagent without any BSA addition was used as a blank. Three microliters of each sample were added to 1 mL of 1X dye reagent and vortexed vigorously. Then, they were incubated at room temperature for 5 minutes. The absorbance were measured at a wavelength of 595 nm using a spectrophotometer (NanoQuant, Infinite M200 PRO Tecan). Sample protein concentrations were read against a standard curve, plotted by using absorbance values of the five BSA standards.

6.2.3.4 Western Blot Analysis of HepG2 cells

Western Blot is a chemiluminescence immunoassay method which is used to identify and quantify target proteins. Briefly, electrophoresis is run through a gel to separate the proteins of interest according to their size. Then, proteins are moved onto a polyvinylidene-fluoride (PVDF^{TM}) membrane by using electric current. The proteins of interest, immunoblotted with specific antibodies, are illustrated by using a colorimetric detection technique. The method is described in detail below.

6.2.3.4.1 Protein sample preparation

Protein samples were kept at room temperature until thawed. Then, equal amount (20-40ug) of quantified protein samples were added to 1.5 ml eppendorfs containing 5uL of 5X loading buffer to standardise the volumes (Table 6.1). After that, the samples were denatured and linearized by heating at 95°C for 5 minutes and then cooled down on ice for 2-5 minutes.

Table 6.1 Quantities of reagents in loading buffer for western blotting

Reagent	Quantity	Final Concentration
Tri HCl (pH 6.8)	625 uL	125 mM
10% SDS	500 uL	4%
Glycerol	1000 uL	20% (w/v)
DTT	200 uL	$6.5x10^{-3}$ mM (w/v)
Bromophenol Blue	125 uL	$2.5x10^{-3}$ mM (w/v)
dH_2O	250 uL	N/A

6.2.3.4.2 Protein separation by electrophoresis

The proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to their molecular weight. 4% stacking gel at pH 6.8 and 7.5% or 10% resolving gel (depending on the size of target protein) at pH 8.8, were used. The amounts of reagents used in 2X 1.5mm gels preparation were described in the following tables 6.2 and 6.3.

Table 6.2 Components of resolving gel (7.5% and 10%) for western blotting

Resolving Gel	7.5% Gel	10% Gel
Acrylamide	5.0 ml	6.6 ml
Resolving buffer	5.2 ml	5.2 ml
10% Ammonium Persulphate	100 ul	200 ul
TEMED	20 ul	20 ul
Distilled water	9.4 ml	7.8 ml

Table 6.3 Components of stacking gel (4%) for western blotting

Stacking Gel	4% Gel
Acrylamide	1.3 ml
Stacking buffer	2.5 ml
10% Ammonium Persulphate	50 ul
TEMED	10 ul
Distilled water	6.1 ml

The glass plates were cleaned with 70% ethanol and left them to dry. The gel casting unit was set up according to manufacturer's instruction. The clean glass slides were assembled in the clamp and water was poured in between to ensure no leaks. The prepared separating gel was then poured until two-thirds of 1.5mm glass plates were filled up. Distilled water or 100% ethanol was layered on top ensure the equal level gel casting and kept at room temperature to allow complete polymerization. Once resolving gel was set, the prepared 4% stacking gel was overlaid on top and wells were created by immediately inserting a 1.5 mm thick, 10-wells comb while taking care to minimize the bubble formation in the gel. It normally took about 1 hour at room temperature to complete polymerization. Once set, the comb was removed gently and gels encased in between the glass plates were secured in an electrophorator tank to create two separate reservoirs. After that, 1L of 1X running buffer was poured into the tank, while washing the wells of gel, until gels were completely flooded with the buffer. The prepared samples were centrifuged and they were loaded to individual well, with 5 ul of a rainbow molecular weight marker (14,300-220-000 Da) alongside. The tank was then connected to the electric current using 100V voltage. It usually took

1 or 1 and ½ hours to complete resolution of the samples in the gel which could be monitored by dye fronts running down to the bottom of the gels.

6.2.3.4.3 Electrophoretic transfer of protein (Electroblotting)

After electrophoresis, the gels were carefully removed from the glass plates. The stacking gels were discarded and the remaining resolving gel parts were washed in cold transfer buffer for 10 minutes. Meanwhile, immobilon-P^{TM} PVDF membranes(0.45µm; Millipore, USA) was immersed in absolute methanol briefly for membrane activation and then immediately soaked in cold transfer buffer until use. At the same time, 2 fibre pads and 2 filter papers for each gel were soaked in cold transfer buffer for at least 10 minutes. Firstly, a saturated fibre pad was laid on the black side of transfer cassette, which was overlaid by a filter paper, gel, PVDF membrane, filter paper and a fibre pad in sequential order, avoiding any bubble stacking in between layers. Then, the enclosed cassette was then transferred into an electrotransfer tank containing 1L of 1X transfer buffer and ice pack. Proteins on the gels were transferred electrophoretically at 100V, 1 hour for 1.5mm thick gel. Alternatively, a constant voltage of 25V was used for overnight transfer for high molecular weight peptide transfer. A magnetic stirrer was also put inside the tank to reduce the heat.

6.2.3.4.3.1 Primary antibody detection

Following electroblotting, membranes were taken out from the cassette while trimming to mark orientation and membrane number. They were then incubated with blocking agent (0.2% I-Block in PBS-Tween 20) and placed on a shaker for 1 hr at room temperature or overnight for ~14 hr at 4°C to prevent non-specific protein binding and direct primary antibody binding to the membrane. After blocking, they

were rinsed briefly in 0.1%PBS-T before incubating with primary antibody prepared in 5ml of 0.2% i-Block in PBS-T for another 1 hour at room temperature or overnight at 4°C on an orbital shaker. After that, membranes were washed with 0.1%PBS-T for 3 times, 10 min interval, on the shaker to remove excess primary antibody.

6.2.3.4.3.2 Secondary antibody detection

After primary antibody application, membranes were submerged again in secondary antibody conjugated with enzyme such as horseradish peroxidase (HRP) for another 1 hour at room temperature on a shaker. Secondary antibody was made up in 0.2% i-Block in PBS-T. Following incubation, they were washed with PBS-T for 3 times, 10 minutes apart, on the shaker. Secondary antibody is attached to primary antibody which binds to the protein of interest.

6.2.3.4.3.3 Immunodetection

A chemiluminescent detection system, ECL/ECL^{+} (Amersham Pharmacia Biotech, Little Chalfont, UK) was used to detect horseradish peroxidase conjugated secondary antibody. In this reaction, cyclic diacylhydrazide luminol is oxidized to generate light at 428 nm. This oxidation is catalysed by combined HRP and peroxidase, emission of thousands of acridinium ester intermediates per minute. Peroxidase then reacts with these intermediates to produce a high intensity chemiluminescence in slightly alkaline conditions. The light emission can be visualised within 60 minutes by application of Hyperfilm^{TM} (Amersham Pharmacia Biotech, Little Chalfont, UK). After secondary antibody application and PBS washing (120 mM, pH 7.6), membranes were placed in a clear thin film pocket with the surface of blotted protein side-up to allow chemiluminescent reagent exposure. ECL chemiluminescent solution was prepared by

mixture of solution A and solution B at 1:1 ratio (1 ml/membrane). The solution was then poured onto the membranes and the membranes were incubated for 5 min at RT. The excess ECL mixture was removed by wiping out with dry tissue papers on top of the pocket. This plastic pocket with membranes inside was then put inside the X-ray film cassette. In a dark room, a hyperfilm^{TM} was then placed in the cassette on top of membrane pocket for various duration depending on the antibody used (1-60 min) to develop a film. Autoradiographs were finally semi-quantified using 2D densitometry software (Imagequant, GEhealthcare, UK). The band density was then measured by using software (Genesnap, Syngene, UK). The bands were first normalised as a function of the loading control (protein of interest/β-actin) or total expression of the proteins (for phospho proteins), then be converted to fold change compared with controls.

6.2.3.5 RNA extraction, isolation and quantification

Total RNA from HepG2 cell culture were isolated, extracted and quantified according to the following steps.

A column based method (RNeasy lipid tissue kit, Qiagen, UK) was used to extract total messenger RNA (mRNA) from samples in QIAzol according to manufacturer's instructions. Following mRNA columnisation, DNase digestion step was completed by adding the mixture of 5µL DNase enzyme and 5µL reaction buffer the sample and incubating for 15 min at RT to digest any contamination genomic DNA. Then, the reaction was stopped by adding 5 µL of stop solution and the tubes were put on heat block at 70°C for 10 min. Following this, total mRNA was quantified by Nanodrop ND-1000 Spectrophotometer (LabTech, UK) at a ratio of readings at a wavelength of

260 (mainly detected RNA) and 280 nm (detected protein, solvents, salts). This ratio was used to measure purified RNA with the ratio of 1.8-2.0 as acceptable. Phenol contamination was also assessed by using the absorbance at 230 and the 260/230 ratio of 1.8-2.0 as highly purified RNA. The quantified RNA samples were finally stored at -70°C.

6.2.3.5.1 Reverse Transcription of Isolated mRNA from HepG2 cells

Complementary DNA was synthesized from each RNA sample by the addition of Bioscript Reverse Transcriptase (Bioline, UK), random hexamers (Bioline, UK) and 10mM dNTP mix (dATP, dGTP, dCTP and dTTP, Bioline, UK) in accordance with manufacturer's protocol.

An aliquot of 500 µg mRNA from each sample was used as template in cDNA synthesis. In this reaction, Random or Oligo$(dT)_{18}$ hexamer attached to its complementary region on mRNA and cDNA was reverse transcribed using dNTP as precursors. The steps involved in cDNA making was described in details as follows.

Firstly, each aliquot was pipetted to a sterile RNase-free microcentrifuge tube together with 1ul each of random hexamer and dNTP mix. DEPC-treated water was then added to standardize a final volume of 10 uL. The mixture was then incubated at 70°C for 10 min, followed by chilling on ice for 1 min. Meanwhile, the reaction premix was prepared by mixing appropriate volumes of the required reagents described in table. After a careful vortex, 10µL of the reaction premix were added to each sample. Then, the samples were mixed gently by pipetting and incubated at 25°C for 10 min followed by 42°C for 30 min. The final cDNA samples were kept at -20°C for further use.

6.2.3.6 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Quantitative real-time PCR analysis was used to validate the mRNA expression levels of selected genes using an ABI 7500 real-time PCR Sequence Detection system. Real-Time PCR was done using 96 well plates, by addition of 24uL of a reaction buffer which is a mixture of Taqman universal PCR master mix (Applera, UK), nuclease-free water, a primer and 18s. Pre-optimized quantitative primer and probe sequences for genes were utilized (Applera, Cheshire, UK). All reactions were multiplexed with the housekeeping gene 18S, provided as a pre-optimized control probe (Applera), enabling data to be expressed in relation to an internal reference to allow for differences in relative threshold efficiency. The PCR steps started with 50°C for 2 min, 95°C for 10 min, 44 cycles of 95°C for 15 min and 60°C for 1 min. Data obtained as cycle threshold (Ct) values according to the manufacturer's guidelines (the cycle number at which logarithmic PCR plots cross a calculated threshold line) were used to determine ΔCt values (ΔCt=Ct of 18S housekeeping gene subtracted from Ct of gene of interest). Measurements were carried out as triplicates for each sample. To exclude potential bias due to averaging data which had been transformed through the equation $2^{-\Delta\Delta Ct}$, all statistics were performed at the ΔCt stage.

6.2.3.7 Statistics

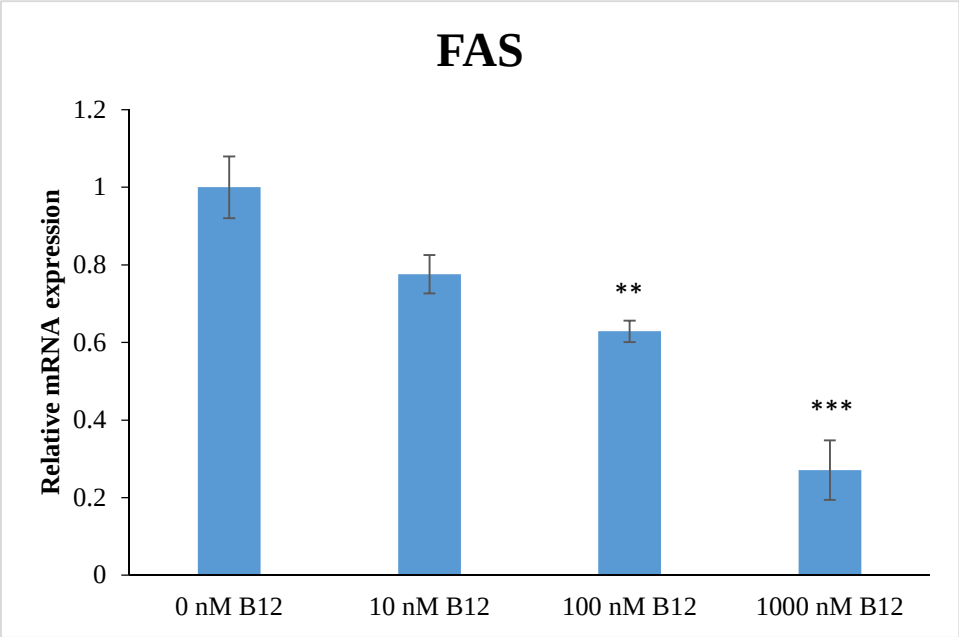
For all analysis, data were expressed as mean±standard error of the mean (SEM) for three independent experiments done in triplicates at least to ensure replicability. A two-tailed unpaired Student's t test was used to analyse the data between two groups, using 0 nM vitaminB12 as a reference group. 'p' value <0.05 were considered to be statistically significant.

6.3 Results

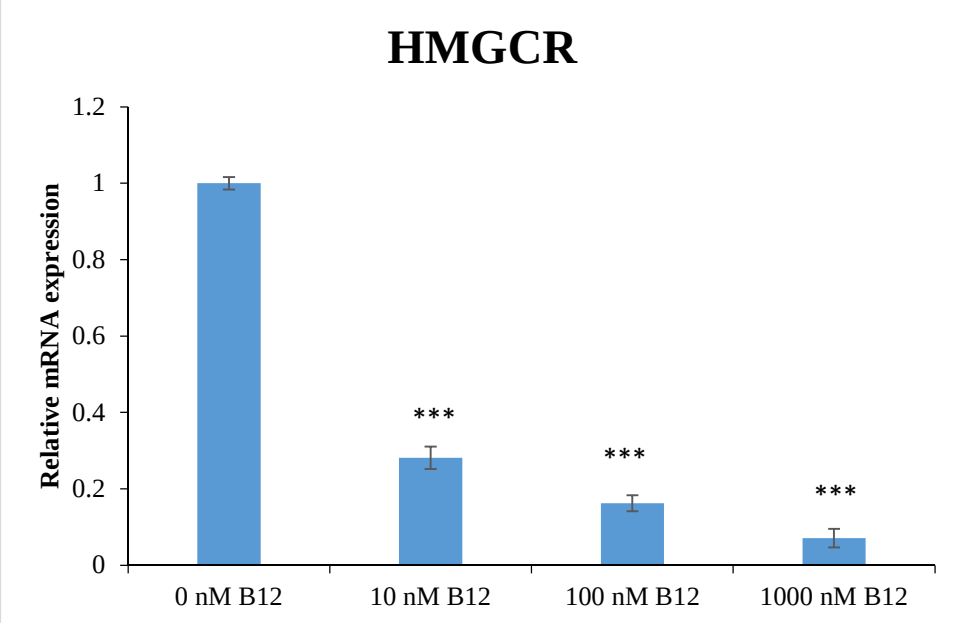
6.3.1. Lipogenic genes were down-regulated by vitamin B12 supplementation in hepatocyte cell line cultured in long-term low vitamin B12 (0nM) media

The mRNA expression levels of key enzymes of triglyceride and cholesterol biosynthesis (FAS and HMGCR, respectively) (Figure 6.1.A and B) and their regulatory genes (Sterol regulatory element binding factors (SREBFs)) were measured by qRT-PCR. SREBFs are conditional transcriptional factors resided in endoplasmic reticulum and can be stimulated when intracellular lipid levels are low (SREBF1 mainly for triglyceride and SREBF2 mainly for cholesterol). When activated and released by protease, nuclear SREBFs bind to the promotor region of more than 30 target enzymes dedicated to increased synthesis and uptake of lipids including FAS and HMGCR. We have found that vitamin B12 supplementation significantly down-regulated the mRNA expressions of FAS and HMGCR enzymes in 100 and 1000nM vitamin B12 conditions compared to 0nM vitamin B12 condition (Figure 6.1 A and B). Furthermore, their transcriptional regulators such as SREBF1 and SREBF2 also showed decreased expression with increasing vitamin B12 concentrations with the lowest in 1000nM B12 condition (Fig 6.1 C and D).

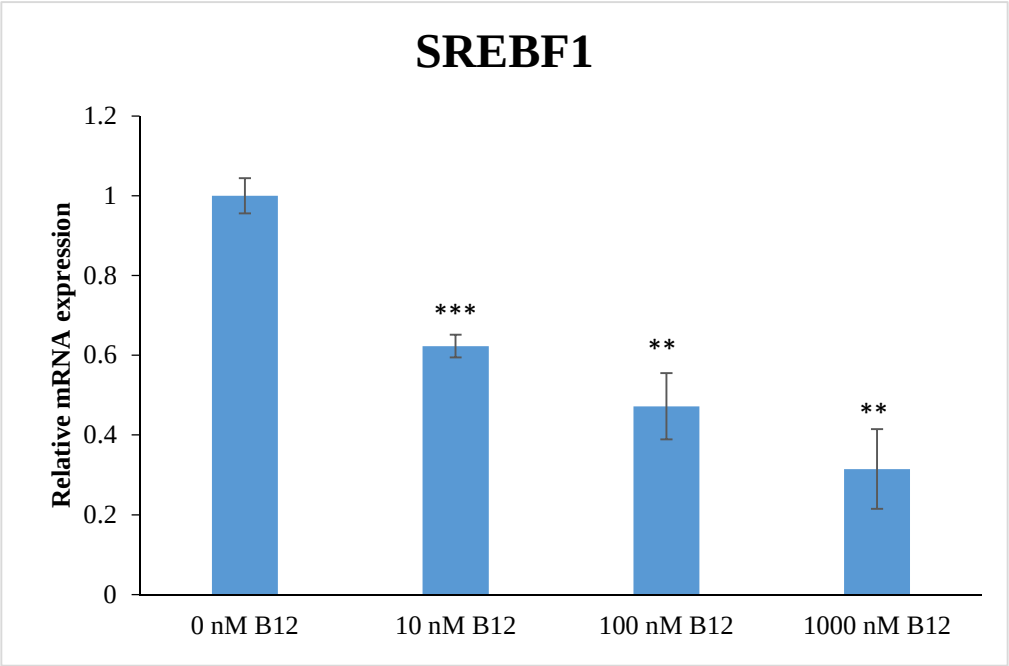
A) Fatty Acid Synthase (FAS)



B) 3-Hydroxy-3-Methylglutaryl-CoA Reductase (HMGCR)



C) Sterol Regulatory Element Binding Factor -1 (SREBF1**)**



D) Sterol Regulatory Element Binding Factor -2 (SREBF2)

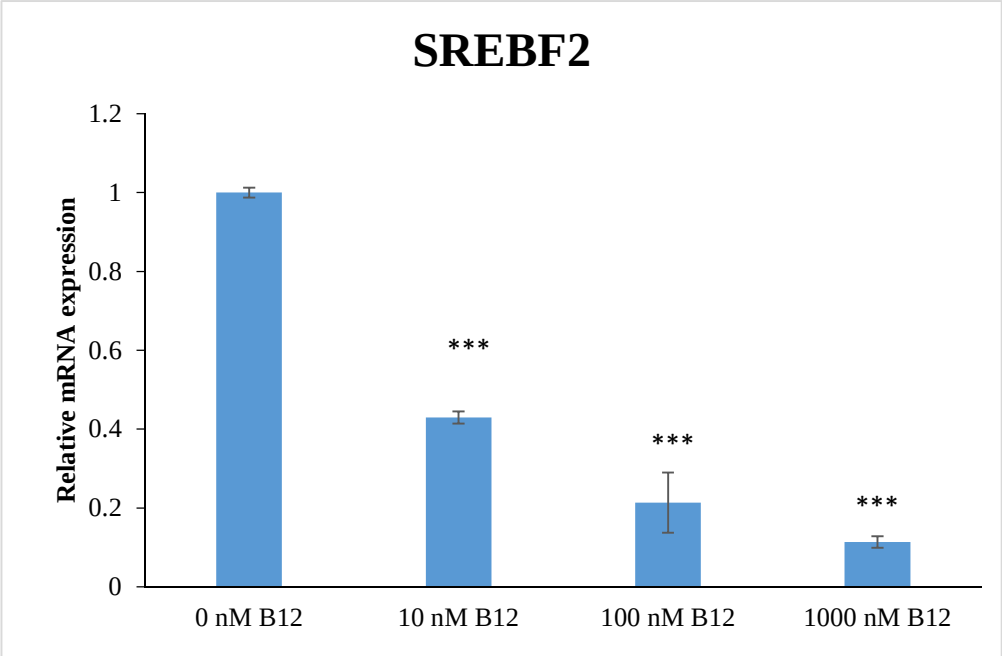
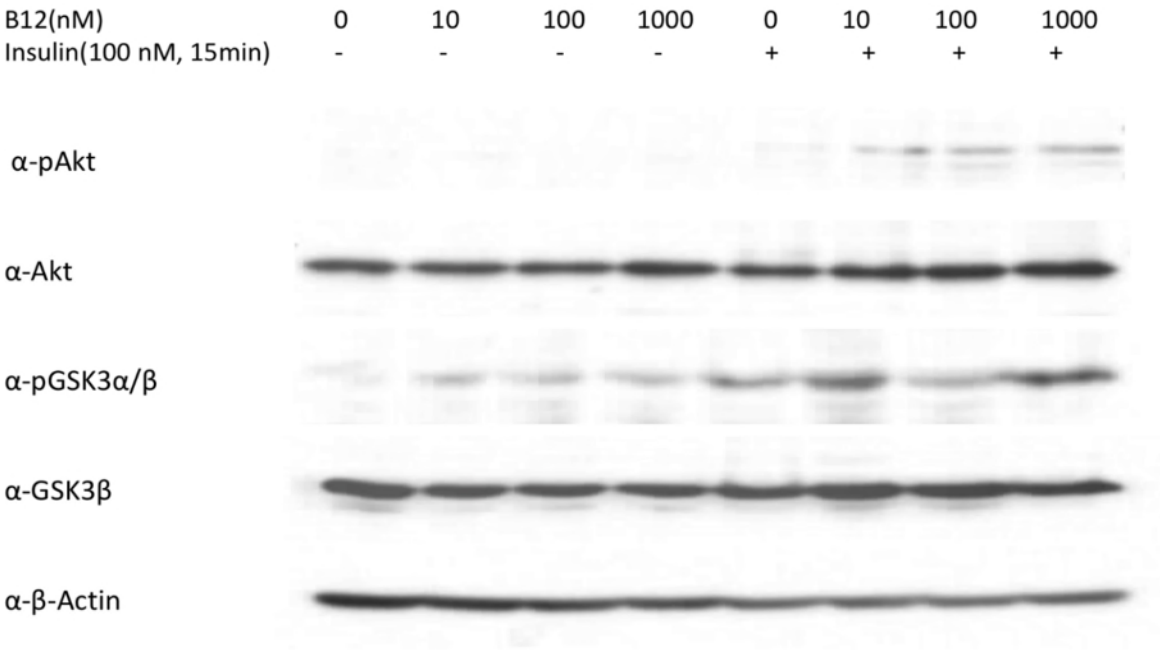


Figure 6.1. Effects of long-term Vitamin B12 supplementation on mRNA expression of lipogenic genes (FAS, HMGCR) and their regulatory genes (SREBF1, SREBF2) in hepatocytes. The mRNA levels was determined by qRT-PCR (n=6). Values are presented as mean±SEM expressed as a relative fold change to control, (0nM Vitamin B12), which was regarded an arbitrary value of 1. p-values *$p<0.05$, **$p<0.01$ and ***$p<0.001$ by student's 't' test. A. Fatty acid synthase (FAS) B. 3-Hydroxy-3-Methylglutaryl-CoA Reductase (HMGCR) C. Sterol Regulatory Element Binding Factor-1 (SREBF1) D. Sterol Regulatory Element Binding Factor-2 (SREBF2)

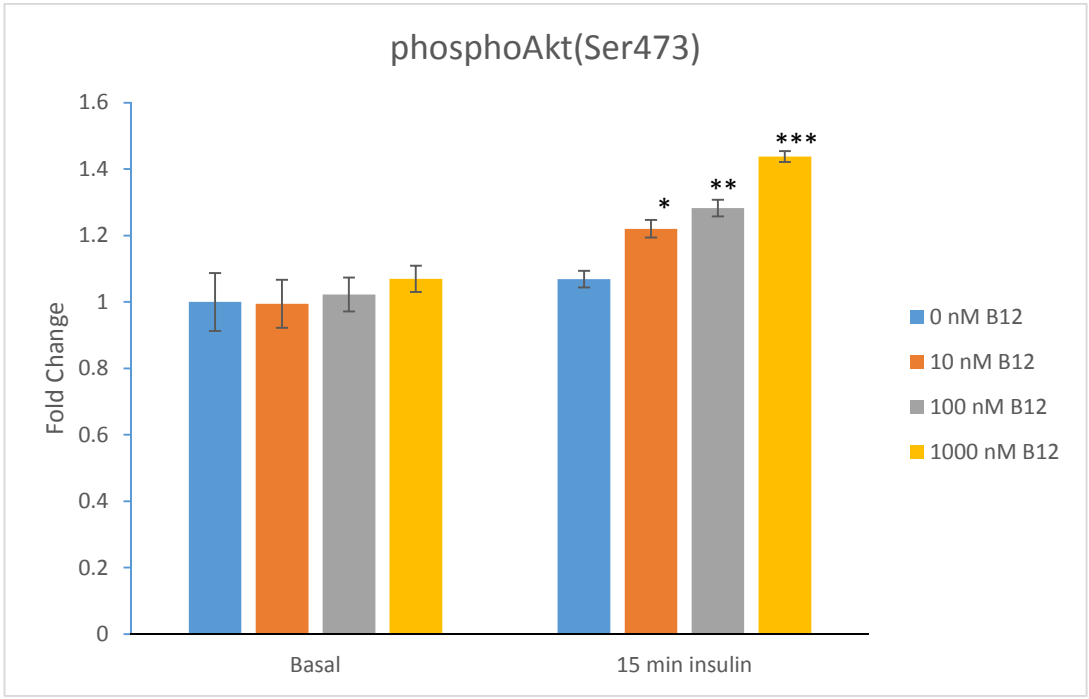
6.3.2. Vitamin B12 supplementation increased insulin signalling in hepatocyte cell lines

Protein expression of markers in insulin signalling pathways were measured in response to insulin (100nM for 15minutes). Once insulin receptors activated, it acts as tyrosine kinase which subsequently activates the docking molecule - insulin receptor substrate (IRS) and protein kinase B (PKB)/Akt. Akt is a serine/threonine specific protein kinase which phosphorylates its downstream, glycogen synthase kinase(GSK)3β, and inhibits its activation of glycogen synthase enzyme and thereby, stimulating glycogenesis. The phosphorylated Akt and its downstream signal, GSK3α/β, were analysed using phospho-Akt (Ser473) and phospho- GSK3α/β (Ser21/9) antibodies. Insulin-induced phosphorylated signals of Akt and GSK3α/β were significantly increased in 10, 100 and 1000 nM vitamin B12 conditions compared to low B12 (0nM) condition. (Figure 6.2). Protein expression of total Akt and GSK3α/β or β-action were found to be no change.

A) Insulin signalling pathway



B) Quantification of pAkt/α-Akt protein bands



C) Quantification of pGSK3α/β/α-GSK3β protein bands

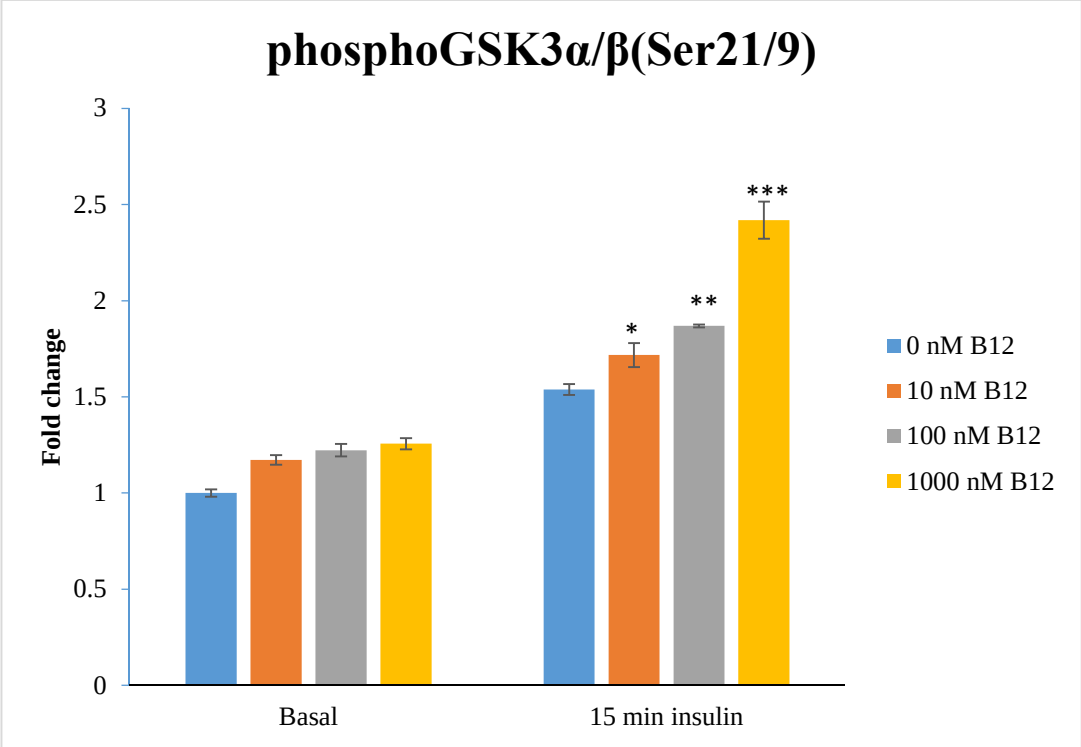
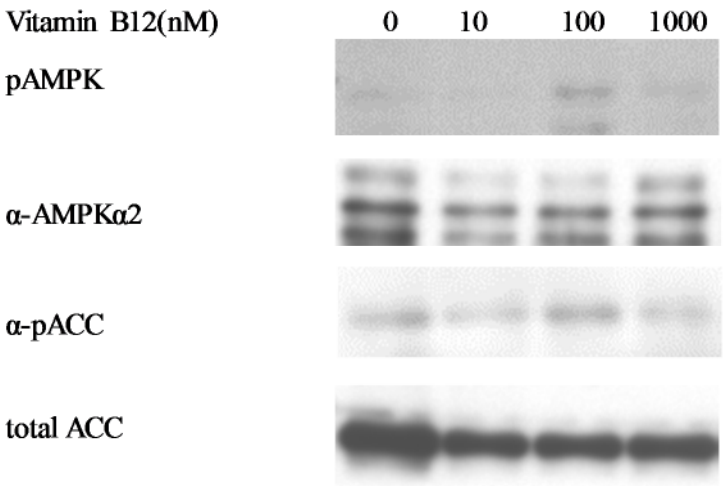


Figure 6.2: Effects of long-term Vitamin B12 supplementation on protein expression levels of pAkt (Ser473) and GSK3α/β (Ser21/9) in hepatocytes, as detected by Western blot (n=4). Values are presented as mean±SEM expressed as a relative fold change to control, (0nM Vitamin B12), which was regarded an arbitrary value of 1. p-values *p<0.05, **p<0.01 and ***p<0.001 by student's 't' test. A. A representative Blot from 3 independent experiments is shown. B and C. Quantification of bands by densitometry.

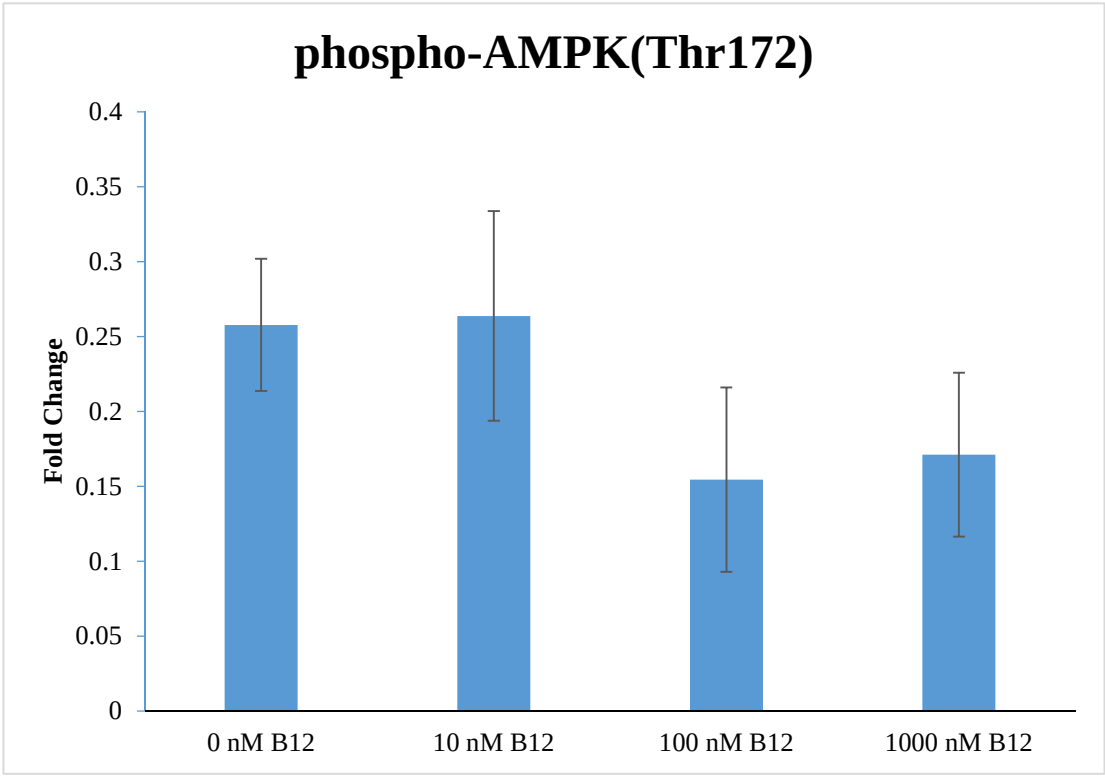
6.3.3. There was no change in the phosphorylation of AMPK and ACC with vitamin B12 supplementation but the protein levels of total ACC were down-regulated with vitamin B12 supplementation.

Western blot analyses of molecules in phosphorylated AMPK and ACC molecules were measured in vitamin B12-treated HepG2 cells. Normally, AMPK activation maintains cellular energy balance by stimulating catabolic pathways such as fatty acid oxidation while preventing energy-consuming anabolic pathways including lipogenesis through inhibiting ACC, FAS and HMGCR enzyme activities. Once activated by phosphorylation at Thr-172 of AMPK α subunit, it subsequently phosphorylates and inactivates, ACCα (Ser79), a rate-limiting enzyme for triglyceride metabolism. ACC is a bidirectional enzyme and determine the fate of fatty acids. If phosphorylated, it favours fatty acid towards beta-oxidation pathway whereas non-phosphorylated form favours formation of malonyl-CoA and lipogenesis. Total expression of AMPK protein was reprobed with AMPKα2 antibody. Total ACC protein was determined with ACCα/β antibody. In HepG2 cells passaged repeatedly in different B12 concentrations for 22 days, there were no change in phospho-to-total AMPK, phospho-to-total ACC ratio and phospho-ACC by total-AMPK ratios. Interestingly, there was decreased protein expression of total ACC enzymes with vitamin B12 supplementation (Figure 6.3).

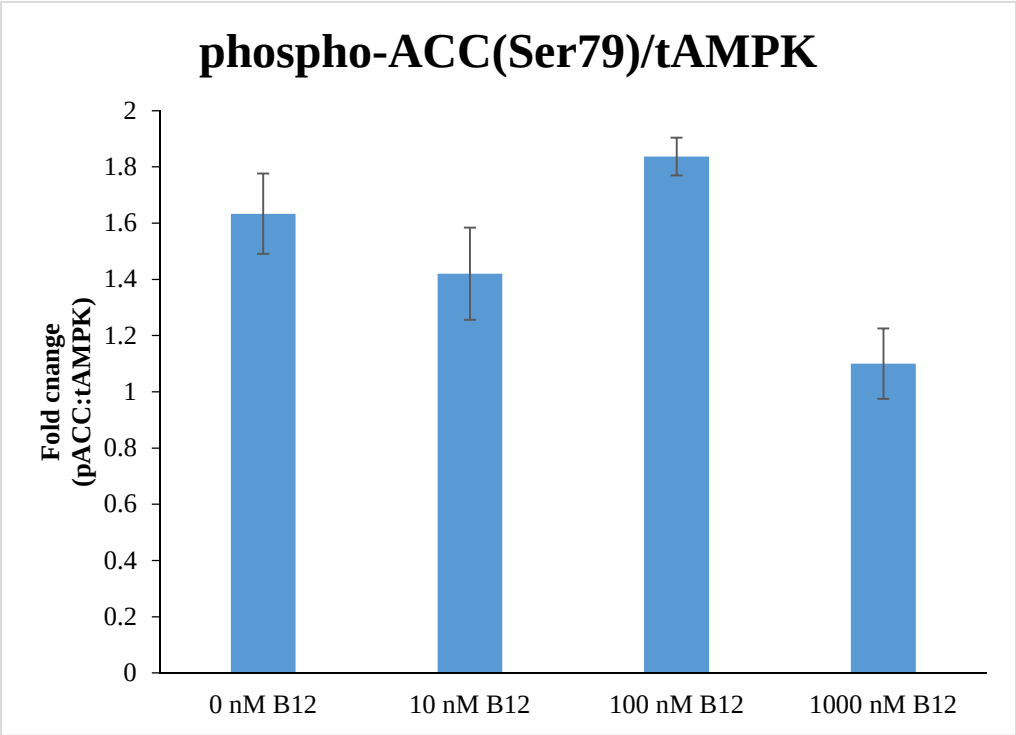
A) pAMPK signalling



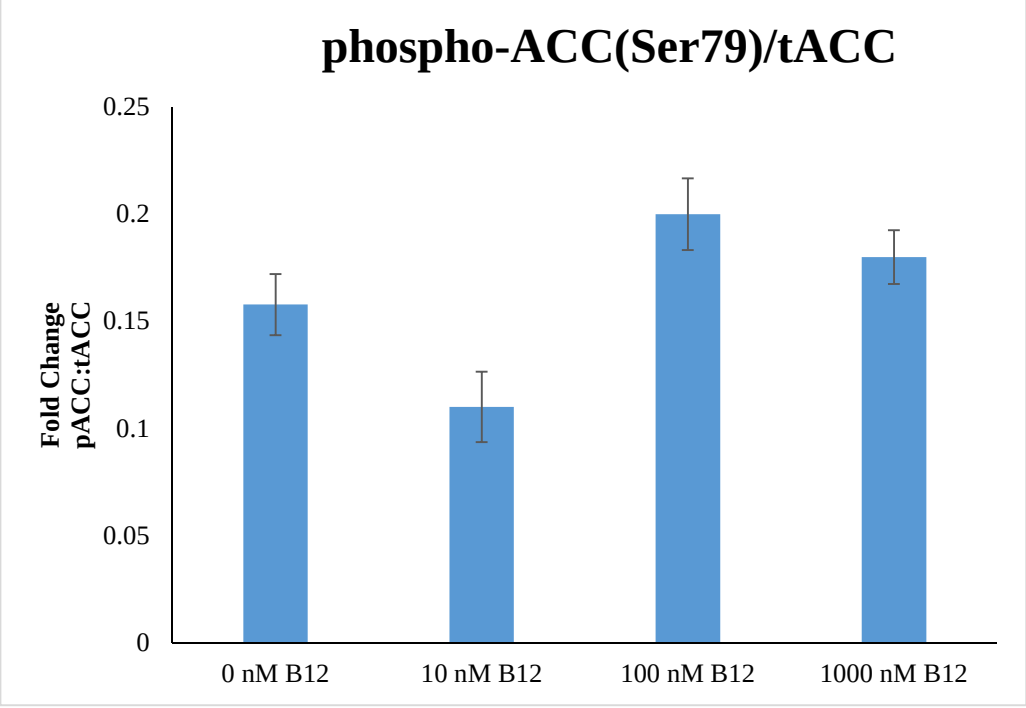
B) Quantification of pAMPK/α-AMPKα2 protein bands



C) Quantification of pACC/α-AMPKα2 protein bands



D) Quantification of pACC/total ACC protein bands



E) Quantification of total ACC protein bands

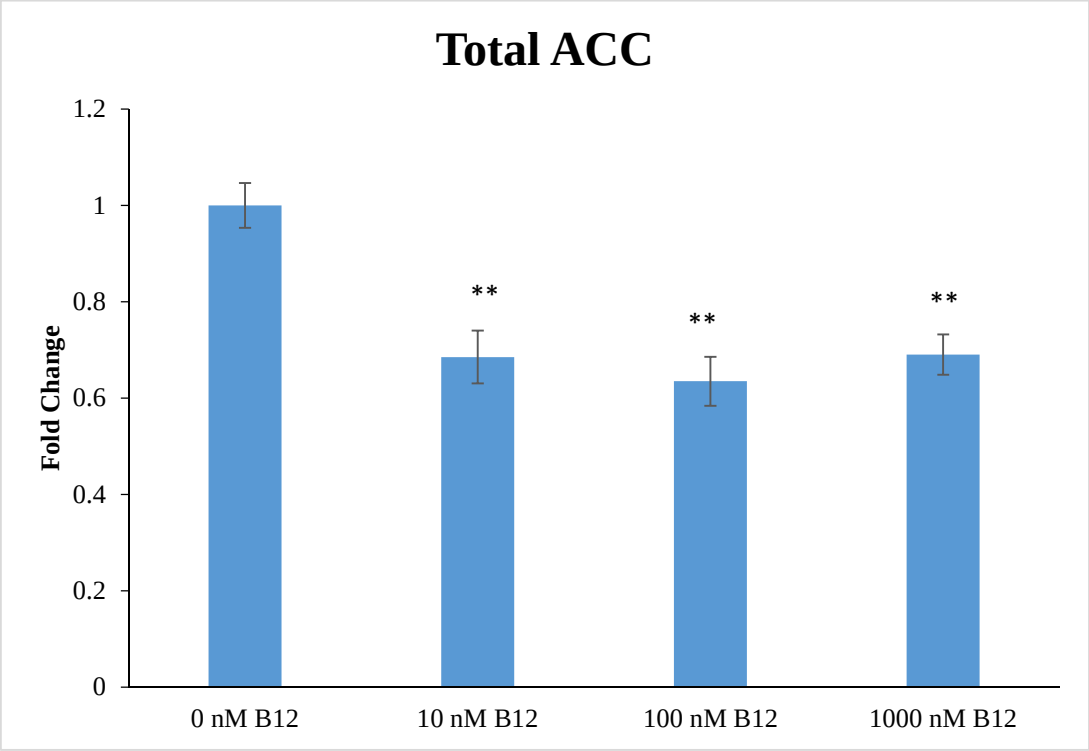


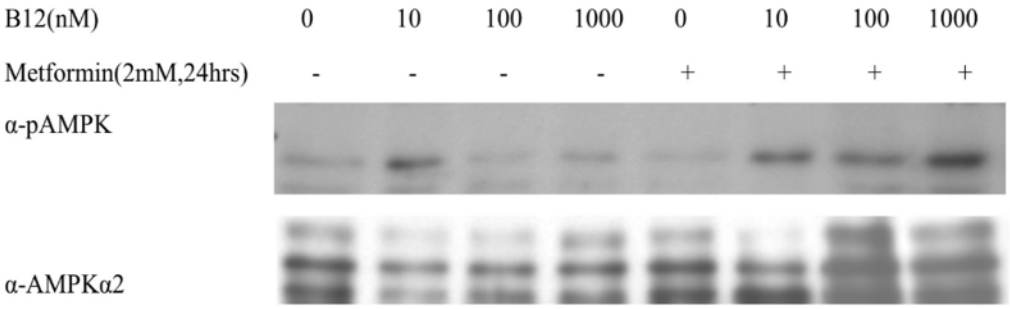
Figure 6.3: Effects of long-term (24 days old) Vitamin B12 supplementation on protein expression levels of pAMPK (Thr172) and pACCα (Ser79) in hepatocytes, as detected by Western blot (n=4). Values are presented as mean±SEM expressed as a relative fold change to control, (0nM Vitamin B12), which was regarded an arbitrary value of 1. p-values *p<0.05, **p<0.01 and ***p<0.001 by student's 't' test. A. Representative Blot from 3 independent experiments is shown. B, C, D and E. Quantification of bands by densitometry.

6.3.4. Phosphorylation of AMPK and ACC enzymes induced by metformin was increased in vitamin B12 supplemented culture compared to low B12 culture (0nM)

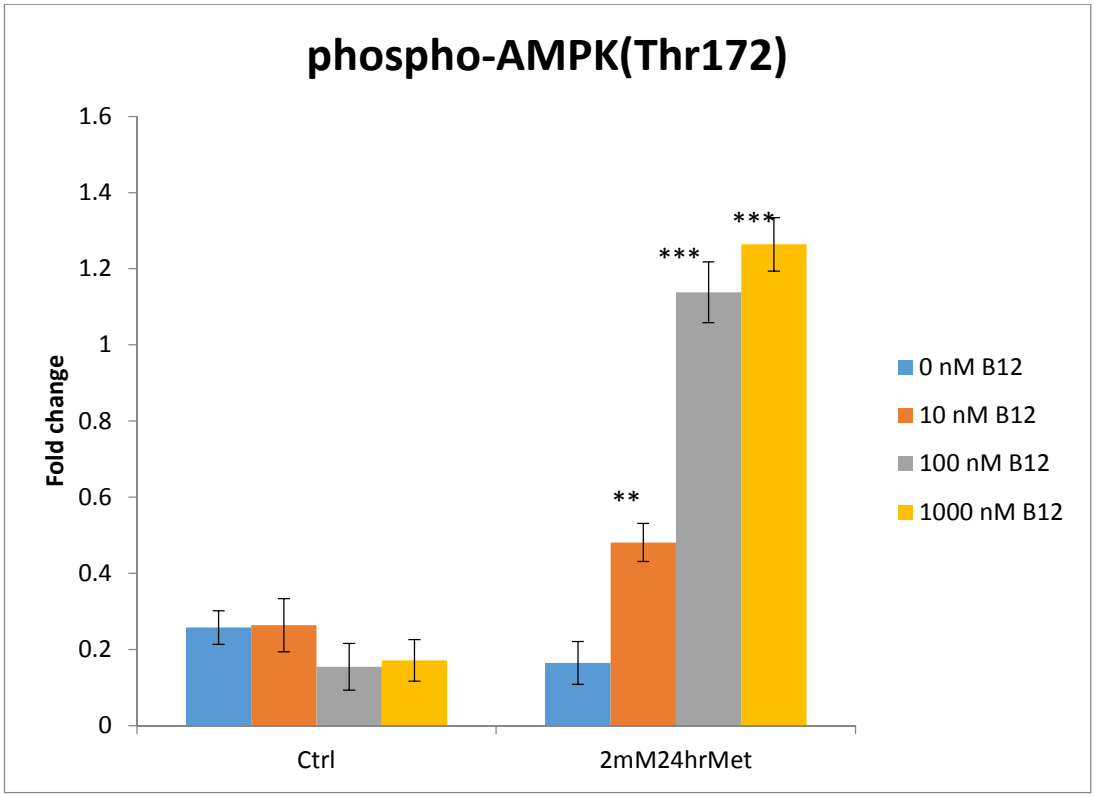
Zhou *et al* have reported that metformin works by activating AMPK in liver. Metformin partially blocks the complex I of mitochondrial oxidative respiration thereby, altering AMP:ATP ratio which phosphorylates AMPK at threonine 172 (Zhou *et al* 2001). Subsequently, the target enzymes of AMPK, such as ACC, FAS and HMGCR, are suppressed and intracellular lipid levels are decreased. Metformin-induced phosphorylation of AMPK is reported to be the greatest with 2mM metformin for 24 hours treatment. We found that the protein expression levels of metformin-stimulated phosphorylated AMPK and ACC ratios were lowest in 0nM vitamin B12 condition compared to other vitamin B12 concentrations (10, 100, 1000 nM) (Figure 6.4). Moreover, protein expression levels of total ACC enzymes were decreased with vitamin B12 supplementation in both basal and metformin-treated conditions, with the lowest in 1000nM vitamin B12 culture treated with metformin. There was no change in total ACC protein expression with or without metformin treatment (Figure 6.4E). As total ACC protein expression levels varied with vitamin B12 concentrations, phosphorylated ACC protein levels were quantified using total AMPK as denominator. Expectedly, phospho-ACC levels were found to be decreased in low (0 nM) B12 compared to 10, 100 and 1000 nM B12 conditions (Figure 6.4 F). Moreover, the down-regulated mRNA levels of FAS and HMGCR together with their transcriptional factors, SREBF1 and SREBF2 respectively, induced by vitamin B12 supplementation were significantly further decreased with metformin stimulation significantly compared to their vitamin B12 counterparts (Figure 6.4. G, H, I and J).

Surprisingly, among 4 vitamin B12 treated conditions, the changes of these enzymes by metformin was highest and most significant in low (0nM) vitamin B12 condition.

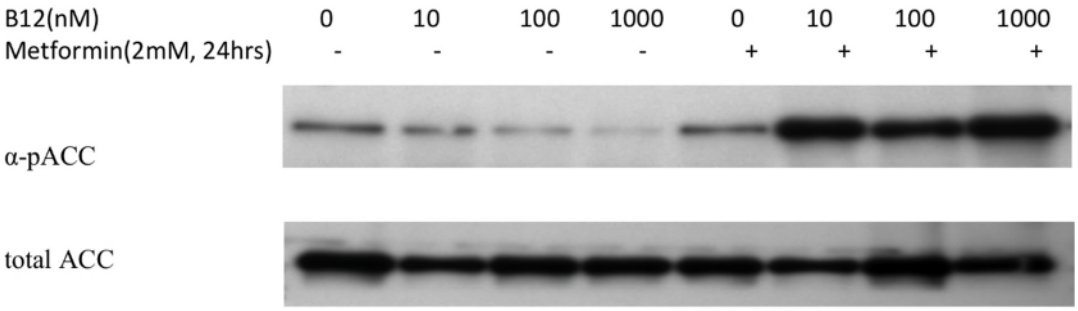
A) pAMPK phosphorylation by metformin



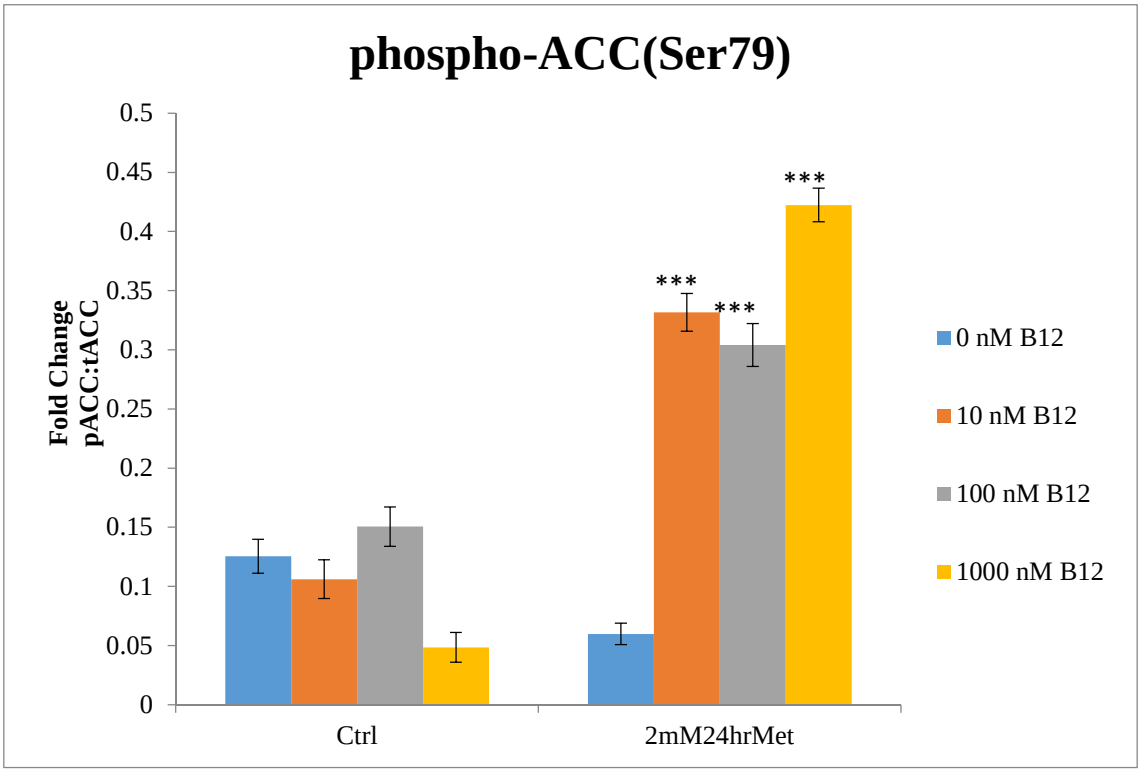
B) Quantification of pAMPK protein band induced by metformin



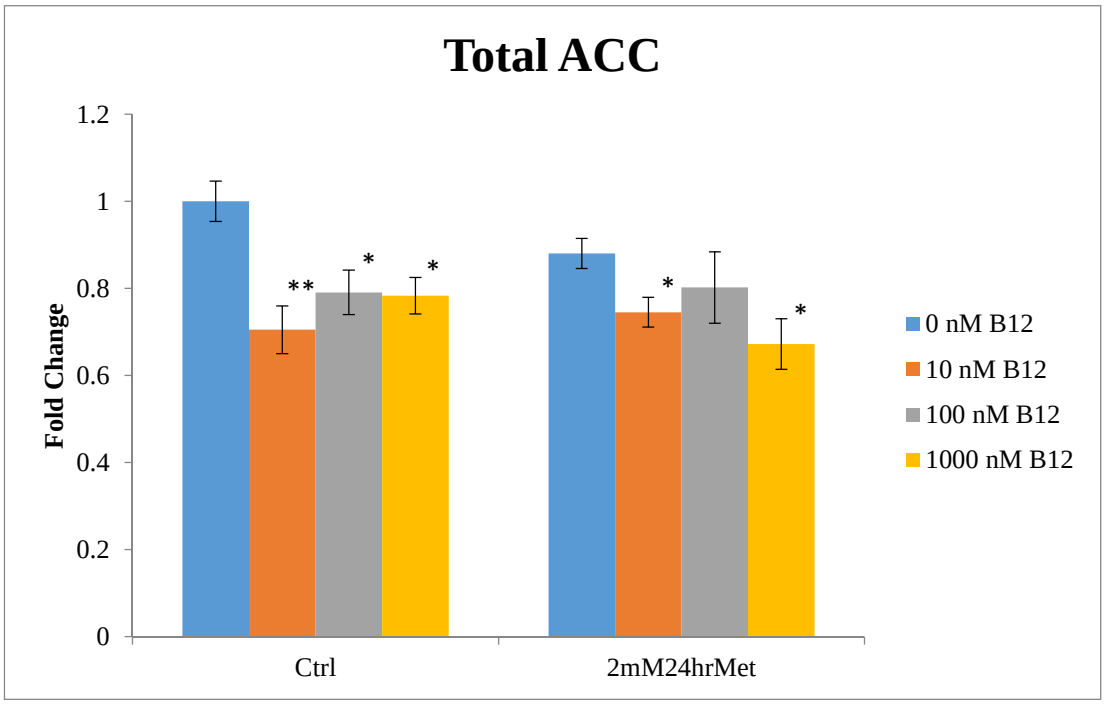
C) pACC phosphorylation by metformin



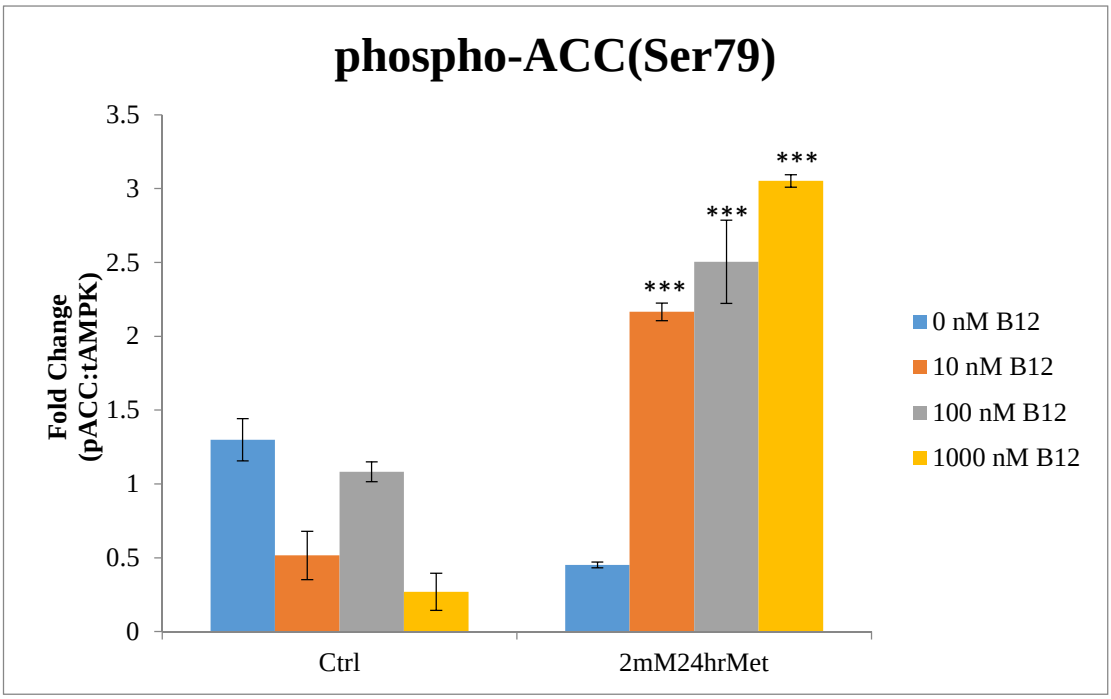
D) Quantification of pACC protein band induced by metformin



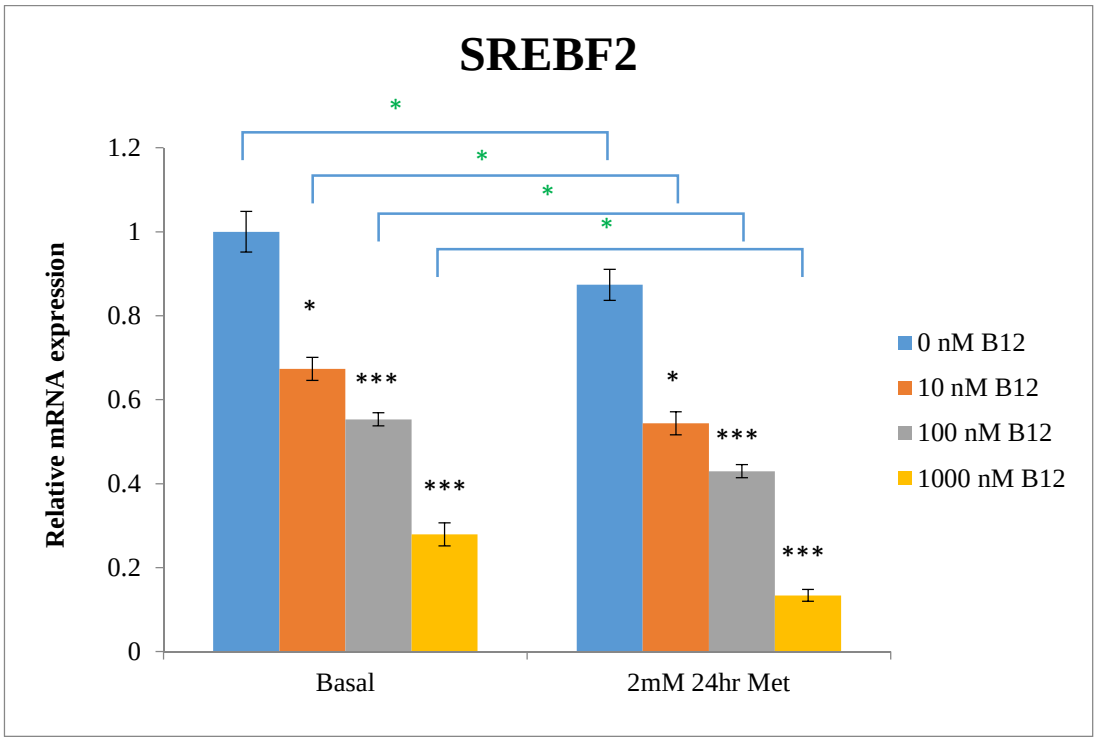
E) Quantification of total ACC protein band



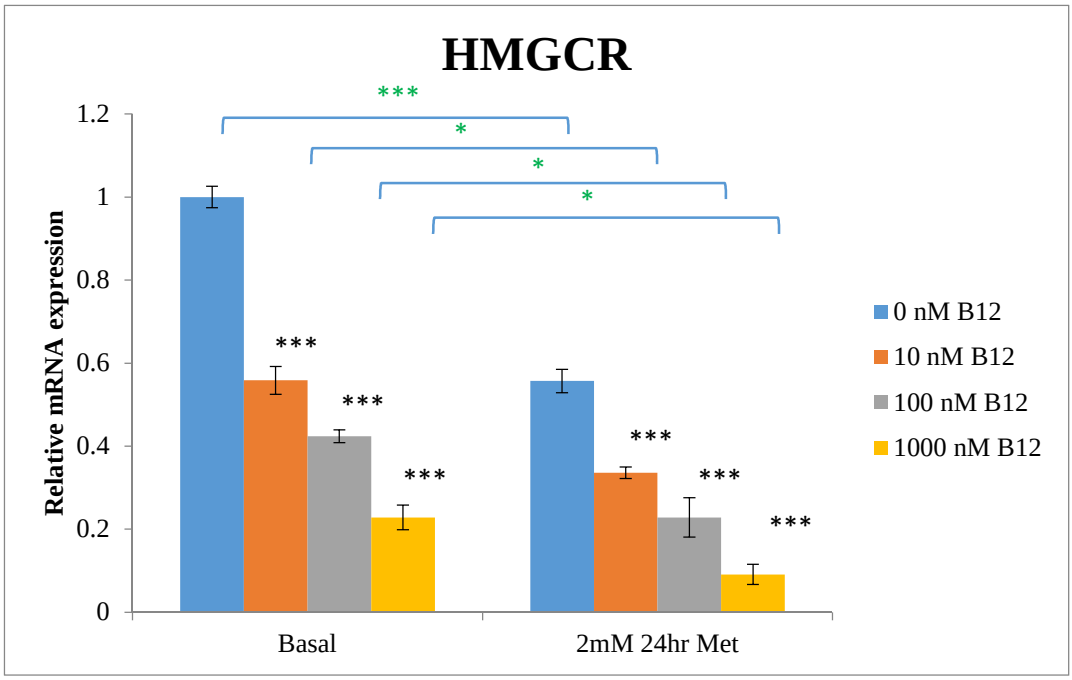
F) Quantification of pACC/total AMPK protein band induced by metformin



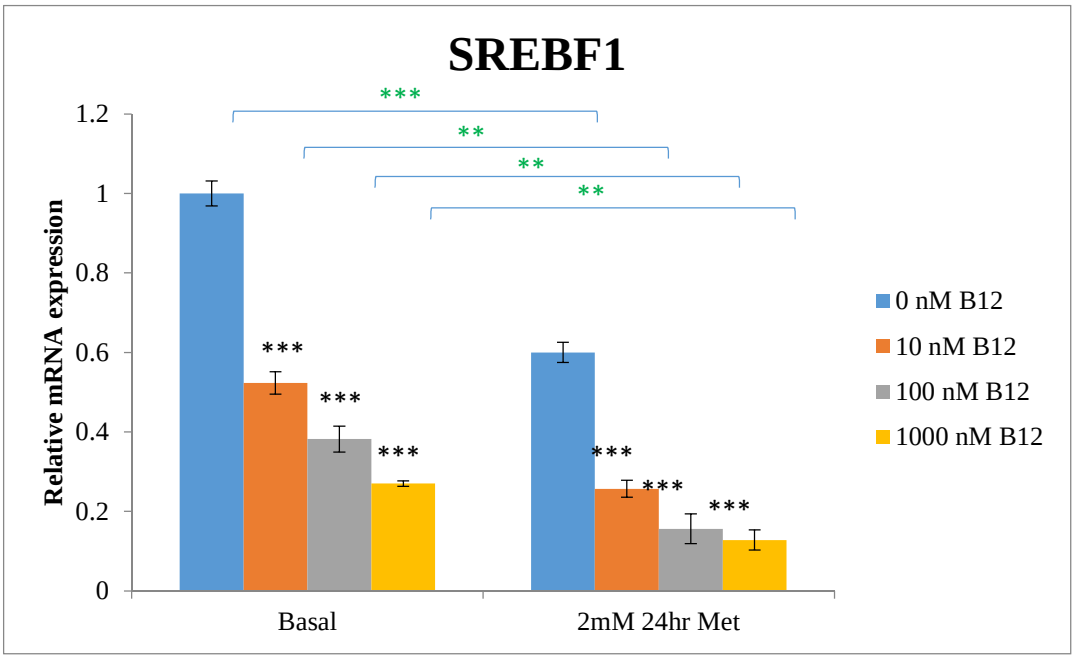
G) SREBF-2 gene expression induced by metformin



H) HMGCR gene expression induced by metformin



I) SREBF1 gene expression induced by metformin



J) FAS gene expression induced by metformin

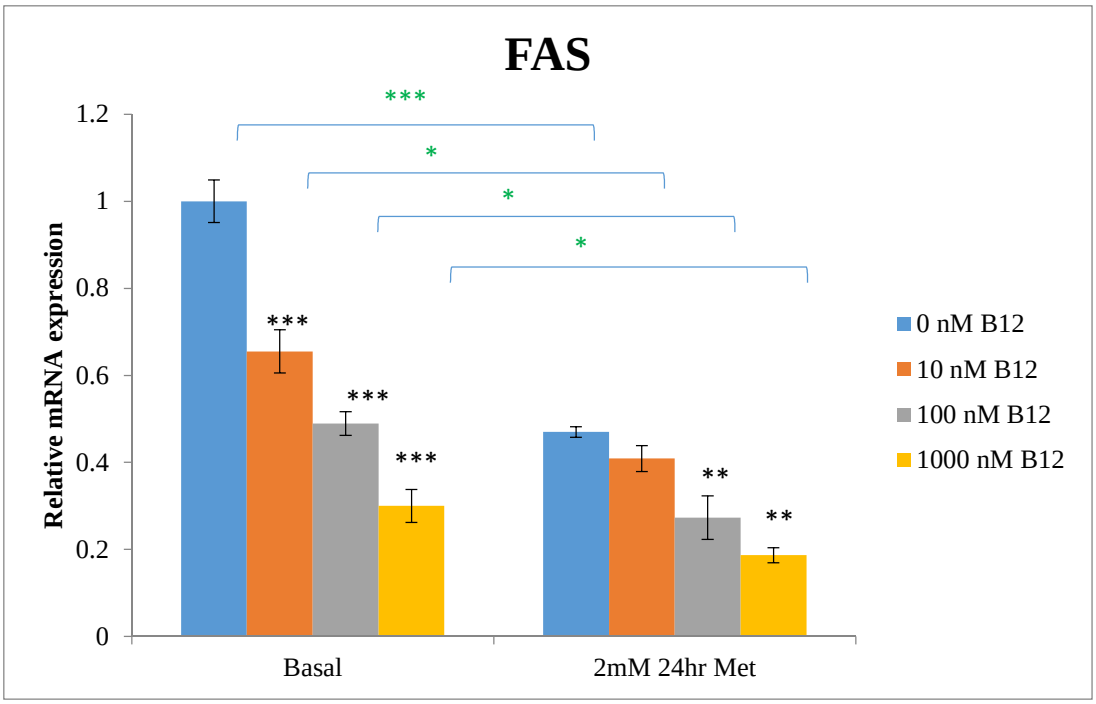
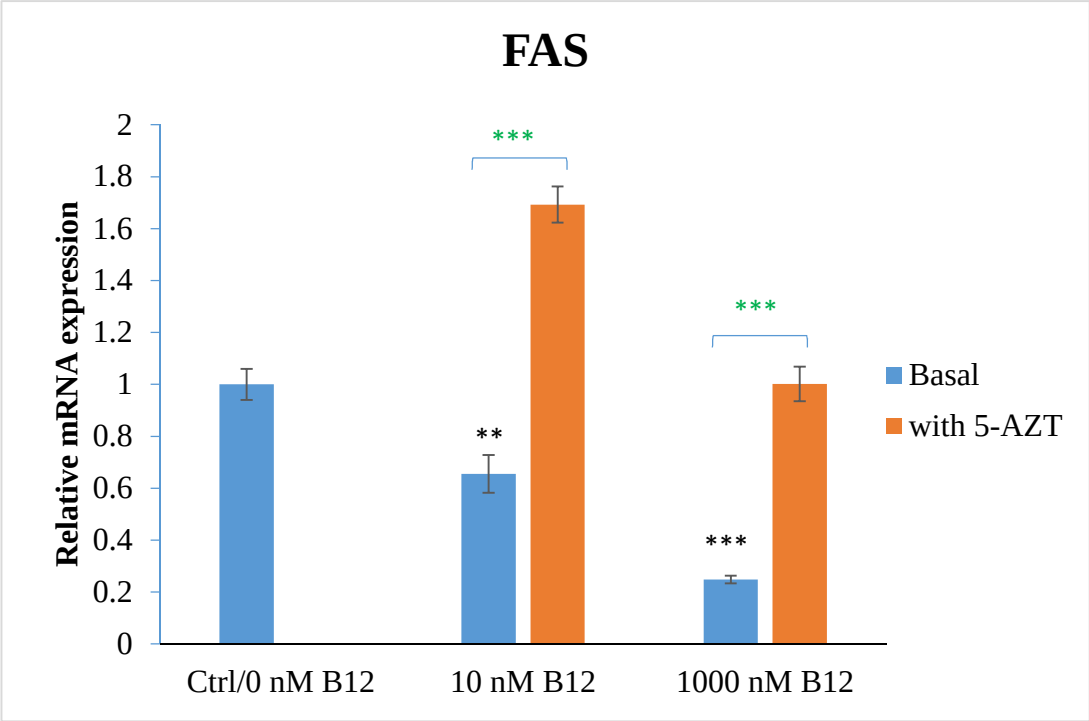


Figure 6.4: Effects of metformin on protein expression levels of pAMPK (Thr172) and pACCα (Ser79) as well as mRNA expression levels of lipogenic enzymes (FAS, HMGCR) and their regulatory genes (SREBF1, SREBF2) in hepatocytes. Protein levels were detected by Western blot (n=4) and mRNA levels were determined by qRT-PCR (n=6). Values are presented as mean±SEM expressed as a relative fold change to control, (0nM Vitamin B12), which was regarded an arbitrary value of 1. The levels of metformin treatment were compared against their vitamin B12 counterparts(*). p-values *p<0.05, **p<0.01 and ***p<0.001 by student's 't' test. A and C. Representative Blot from 3 independent experiments is shown. B, D, E and F. Quantification of bands by densitometry. G, H, I, J. mRNA expression by PCR

6.3.5. Down-regulation of lipogenic enzymes by vitamin B12 supplementation were blocked with anti-methylating agent, 5-azacytidine (AZT)

5-Azacytidine is a cytosine analogue, impeding DNA methylation and thereby re-expression of hyper-methylated genes. Adipocyte culture with vitamin B12 has been reported that vitamin B12 deficiency alters the methylation potential of the cells and induces increased expression of cholesterol biosynthetic enzymes due to hypomethylation. To validate the role of vitamin-B12 related methylation in the regulation of lipogenic gene expression, mRNA levels of HMGCR and FAS were measured by qRT-PCR after differentiating the B12-supplemented cells (10 nM, 1000 nM) vitamin B12 in media with methylation blocker/AZT (300nM, 3 days). AZT significantly increased the expression levels of lipogenic genes (FAS and HMGCR) down-regulated by long-term vitamin B12 culture (Figure 6.5).

A) FAS gene expression by AZT treatment



B) HMGCR gene expression by AZT treatment

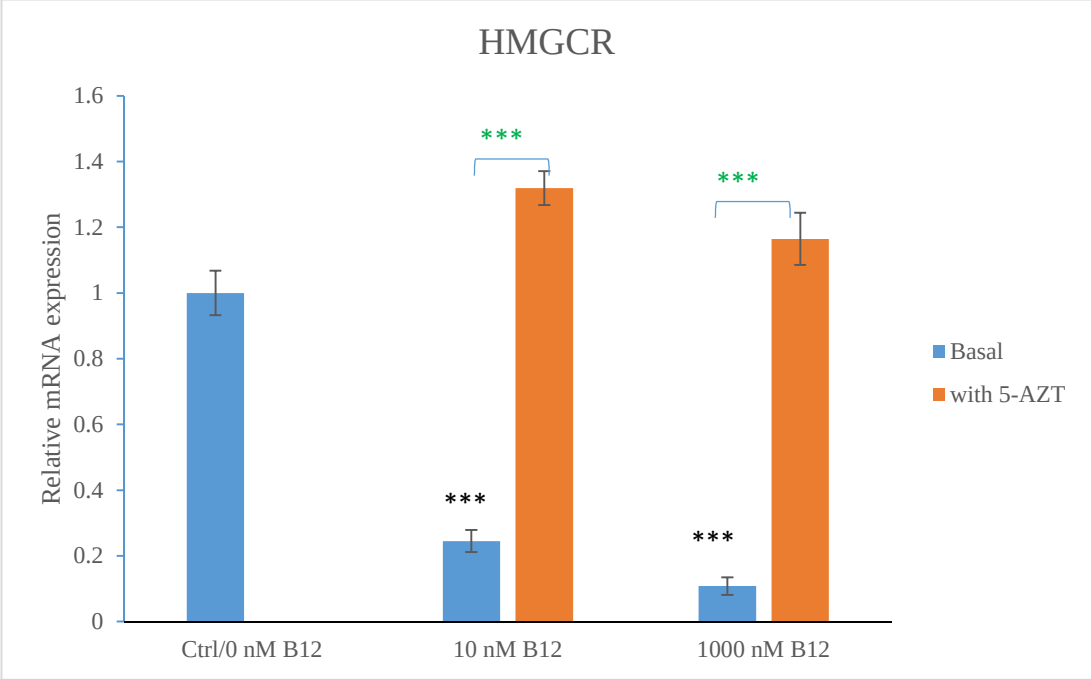


Figure 6.5 (A and B): Effects of Vitamin B12 on mRNA expression levels of hepatic lipogenic genes (FAS, HMGCR) in the presence of methylation blocker (5-azacytidine). The mRNA levels was determined by qRT-PCR (n=3). Values are presented as mean±SEM expressed as a relative fold change to control, (0nM Vitamin B12), which was regarded an arbitrary value of 1. The levels of AZT treatment were compared against their vitamin B12 counterparts (*). p-values *$p<0.05$, **$p<0.01$ and ***$p<0.001$ by student's 't' test.

6.4 Discussion

The association of vitamin B12 deficiency with blood dyslipidaemia has been reported by several clinical studies (Mahalle *et al* 2013; Adaikalakoteswari *et al* 2014; Adaikalakoteswari *et al* 2015a: Adaikalakoteswari *et al* 2015b). The proposed mechanistic model is that it occurs through reduced methylation potential and hypo-methylation of lipogenic enzymes in vitamin B12 deficient condition and which has partly elucidated by a study done on adipocyte culture (Adaikalakoteswari *et al* 2015a). As liver plays a central role in both uptake and transport of lipids in the circulation, examining the changes of lipid metabolism in vitamin B12 deficient will enable to understand how serum vitamin B12 deficiency can lead to abnormal lipid profiles and, more importantly, whether vitamin B12 supplementation can reverse this. Similar to the previous adipocyte study (Adaikalakoteswari *et al* 2015a) and in vivo studies (Kumar *et al* 2013), we have found that there was increased expression of hepatic lipogenic enzymes in low vitamin B12 condition due to impaired methylation. The novel finding in this study is that insulin signalling and AMPK signalling pathways can be affected by vitamin B12 deficiency. Consequently, lipid-lowering effects of metformin are found to be impeded in low vitamin B12 cells. Finally, the vitamin B12 supplementation culture model suggests that epigenetic changes due to low vitamin B12 conditions can be countered by successive long-term vitamin B12 supplementation.

6.4.1. Effects of low vitamin B12 levels on lipid synthesis

Firstly, it was decided to look at the expression of enzymes important for cholesterol and triglyceride synthesis in liver cells, which have been passaged in different vitamin

B12 conditions successively for several times. Enzymes for triglyceride were included in this study because liver is central regulator of blood triglyceride levels. Moreover, in Wister rat study done by Kumar and Frenkel groups, it was independently observed that vitamin B12 restriction in mothers increased lipid accumulation in viscera and altered adipose tissue composition in the offspring by increasing the activities of hepatic FAS and ACC (Kumar *et al* 2013). HMGCR and FAS mRNA expression levels were selected to be studied. FAS was chosen rather than ACC the rate-limiting enzyme in triglyceride synthesis because of the limited resources and FAS was readily available in our lab. Moreover, total ACC expression levels were examined with western blotting and FAS is highly correlated with ACC and the rate of fatty acid synthesis (Kumar *et al* 2013). In this study, there was down-regulation of HMGCR and FAS mRNA levels in the liver supplemented with vitamin B12. The decreased lipogenic enzyme levels are in coordination with their transcription factors (SREBF1 and SREBF2). The effects of maternal B12 deficiency on lipid synthesis of progeny's liver have been studied in animals over time and they have consistently reported that maternal vitamin B12 deficiency increased liver lipid content, triglyceride, total cholesterol, cholesterol ester and free cholesterol, in their offspring(Moore *et al* 1962; Armstead *et al* 1971; Icayan *et al* 1962). Furthermore, an adipocyte cell culture model indicated that the cholesterol levels were found to be increased in no or low vitamin B12 conditions (Adaikalakoteswari *et al* 2015a). Accordingly, the possible findings of intracellular lipids in our study could be decreased with vitamin B12 supplementation. At the same time, as vitamin B12 is essential for normal metabolic function in the cells, another possibility is that vitamin B12 supplementation might return the cellular metabolism to normal and the decreased expression of the rate-limiting enzymes in

our study might suggest that the allosteric inhibition of the enzymes by the end products, cholesterol and triglyceride levels. Thus, we would suggest to include the measurements of intracellular lipid content in the future studies.

6.4.2. Impaired methylation due to low vitamin B12 levels implicates the changes in lipigenic gene expression

Next, in order to explore the possible mechanism behind vitamin B12-induced decrease in expression of lipid synthetic enzymes, the effects of vitamin B12 supplementation to the expression levels of these enzymes were studied by adding the methylation blocker, AZT to our HepG2 model with long-term vitamin B12 culture. An increase in expressions of HMGCR and FAS enzymes by AZT was seen in comparison to their basal vitamin B12 counterparts. The change by AZT was higher than low (0nM) vitamin B12 condition, suggesting that the amount of vitamin B12 (120pM) in FBS was high enough to methylate the lipogenic gene expression to considerable extent. Studies of Vitamin B12 as an cofactor for methionine synthase(MS)/methyltransferase in epigenetics have also reported that vitamin B12 helps the stability of MS by translational up-regulation (Oletean *et al* 2003; Gulati *et al* 1999; Kamely *et al et al* 1973). As MS is vital for one carbon change in methylation which is important for normal DNA package, it is presumable that intracellular low B12 levels can induce hypomethylation. Based on this evidence and our findings of AZT treatment, we have postulated that in vitamin B12 deficiency, the DNA sequences are loosely packed because of reduced methylation and this might result in increased gene expression.

6.4.3. Vitamin B12 supplementation improves insulin sensitivity

In addition to the association of dyslipidaemia with vitamin B12 deficiency, there are a number of reports from clinical studies that vitamin B12 deficiency can increase insulin resistance done by HOMA-IR(Yajnik *et al* 2008; Krishnaveni *et al* 2014), for which intracellular mechanism has not yet been confirmed. Thus, we decided to look at the insulin signaling pathway. We have found that HepG2 differentiated in vitamin B12 supplemented culture for prolonged period can increase phosphorylation of Akt, an upstream insulin signal. This effect is followed by increased phosphorylation of GSK enzyme which subsequently inhibits glycogen synthase enzyme and thereby glycogenesis in the presence of insulin. Thus, this might indicate that vitamin B12 supplementation to HepG2 culture improves insulin signaling. In other words, this might also suggest that long-term low vitamin B12 culture induces insulin resistance in HepG2 cells. As previously described in (Chapter 1.4.2), the primary role of liver in lipid metabolism is by regulating synthesis of ApoB100 lipoprotein in the balance of insulin and fatty acid entry. It is stated that in liver with normal insulin sensitivity, there is inhibition of ApoB100 synthesis and thereby suppression of very low density lipoprotein (VLDL) production which represents major endogenous triglyceride compartments in the blood. Although the causal relationship between lipid dysregulation and insulin resistance in vitamin B12 deficient state has not yet been examined extensively, Saravanan and his group suggested that insulin resistance was preceded by intracellular impeded lipid metabolism in vitamin B12 deficiency (Saravanan *et al* 2010). Based on our findings in this chapter, the postulated mechanism of how low vitamin B12 condition leads to dyslipidaemia has been developed. Firstly, in vitamin B12 deficient liver with normal insulin sensitivity, there

might be impaired mobilization of intracellular lipids due to increased biosynthesis and suppression of ApoB100 production at the same time, possibly resulting in liver steatosis. This is supported by a clinical study which observed the significant association of vitamin B12 deficient population with higher risk of non-alcoholic fatty liver disease (NAFLD)(Koplay *et al* 2011). Subsequently after that, large amount of lipid accumulation later induces metabolic stress inside the cell and eventually the cell becomes insulin resistant.

6.4.4. Lipid-lowering effects of metformin in vitamin B12 deficient hepatocytes

At the basal condition without metformin treatment, there was no change in phosphorylated AMPK (Thr172) levels among HepG2 culture with vitamin B12 addition. When metformin was given, the increased phosphorylation of AMPK was observed in all HepG2 cells with additive vitamin B12 culture. This might indicate that metformin-induced AMPK phosphorylation was impeded in low B12 conditions and cells with vitamin B12 supplementation could improve the situation. There are some suggestions on how vitamin B12 deficiency might interfere with metformin action on activation of AMPK. Then, we have looked at metformin effect on the phosphorylation of ACC at Ser79, the downstream signal of AMPK. In line with AMPK phosphorylation, there was no difference in ACC phosphorylation. Similarly, once AMPK activated by metformin, the ACC phosphorylation was found to be increased positively with vitaminB12 concentrations. Previous in-vivo studies have reported that increased levels of total ACC mRNA expression with vitamin B12 deficiency (Khaire *et al* 2015). As the phosphorylation capacity of AMPK on ACC also depends on total ACC availability, we also examined the levels of total ACC-1 protein expression. We chose ACC-1 because it is highly expressed in the liver. There

was decrease in ACC total protein expression with vitamin B12 supplementation. Even in semi-quantitative like Western Blot, the effect was quite apparent. We also determined the ratio of ACC phosphorylation levels by dividing with either total ACC protein levels or total AMPK levels and there was increased phospho:total-ACC or phospho-ACC:total-AMPK with increasing B12 conditions. This might suggest that vitamin B12 supplementation could improve the mitochondrial function of hepatocyte which is the primary site of metformin action. Theoretically, in vitamin B12 deficiency, MMA is accumulated and impedes Coenzyme Q biosynthesis. Thus, it is possible that vitamin B12 sufficient culture helps relieve these MMA and thereby enhancing mitochondrial respiration. This work should be further confirmed by future study with vitamin B12 on mitochondrial respiration. It should also be noted that as phosphorylated ACC is the inhibitory form of ACC enzyme which converts acetyl CoA to malonyl CoA, a precursor for triglyceride synthesis. The increased fatty acid oxidation can be explained by the fact that reduced MMA accumulation due to vitamin B12 can improve cytosolic fatty acyl transfer into mitochondria which was previously inhibited at carnityl parmitoyl transferase-1 (CPT-1) by vitamin B12 deficiency. Thus, we would conclude that vitamin B12 supplementation helps improve lipid lowering effects of metformin, possibly by improving energy metabolism within the cells.

We also examined other important downstream enzyme expression of AMPK, important for lipogenesis, FAS and HMGCR together with their transcriptional factors SREBF 1 and 2. AMPK activation by metformin or other drugs/metabolites are reported to reduce the expression of these enzymes with their transcriptional factors in liver (Lee *et al* 2008). Likewise, when metformin was given, we have observed that HMGCR and FAS expression levels were further reduced by metformin treatment and

this was accompanied by reduction of their transcriptional factors. The levels are in accordance with increasing concentrations of vitamin B12. Thus, it is likely that in vitamin B12 deficient cells, metformin-induced lipid lowering effects of metformin can be enhanced by adding vitamin B12.

6.4.5. Strengths and Limitation

The strength of the study is that this is the first study to support that cells with supplemented vitaminB12 can improve the efficacy of metformin on AMPK activation and improve lipid profiles. Moreover, the use of liver cell culture could help extrapolation of the mechanism to clinical studies. Furthermore, the causal relationship of up-regulation of hepatic lipid synthetic enzymes with vitamin B12 deficiency was confirmed by AZT study. In addition, the inclusion of 4 different vitamin B12 concentrations will help for future successive studies. However, these data are supported by long-term vitamin B12 supplementation and thus, the metformin effect on liver cell with shorter duration of treatment would be of advantage before implementing the supplementation of vitamin B12 to vitamin B12 deficient patients at metformin initiation. Although we have reported the AMPK signalling pathway of metformin affected by vitamin B12 concentrations, the findings should be confirmed by inhibitor studies using blockers or transfection of AMPK. Moreover, the finding should be confirmed in animal model of vitamin B12 supplementation with metformin treatment before future implication.

6.4.6. Suggestions for future study

The future study should be expanded by looking at other key enzymes in lipid metabolism such as ACC, HMGCS and LDLR together with intracellular lipid content.

Then, we need to explore the other factors contributing to elevated VLDL outputs such as increased fatty acid influx, mainly from lipolysis of adipocytes. Amstead and his group have predicted that the enzyme for conversion of free to esterified cholesterol might be impaired in vitamin B12 deficiency (Armstead *et al* 1971), possibly suggesting involvement of lipid esterification enzyme, whereas Khaire *et al* has reported that the lipid fractions in the liver are affected by vitamin B12 (Khaire *et al* 2015). Thus, we suggest that the individual fatty acid fractions of increased lipid content(such as total cholesterol and triglyceride levels) due to vitamin B12 addition should be included. In addition, a recent study of vitamin B12 supplementation to wistar rats during pregnancy has suggested that there is increased plasma triglyceride levels in offspring of vitamin B12 supplemented rats with decreased hepatic eicosapentaenoic acid (EPA) accumulation which regulates triglyceride metabolism (Khaire *et al* 2015). Thus, the relationship between omega3 fatty acid with vitamin B12 on lipid metabolism should be further explored. Furthermore, the role of vitamin B12 in mitochondrial dysfunction and metabolic dysregulation need to be fully established. In addition, as the lipid composition of the tissues and the circulation can be differed by gender (Icayan *et al* 1962), it is of importance to report findings from the clinical studies looking at the lipid profile in B12 deficiency by gender.

6.4.7. Clinical implications

Vitamin B12 is vital for normal lipid homeostasis. However, because of high storage in liver and low prevalence of vegetarian population, its significance is usually ignored. On the other hand, having aware of importance of folic acid in neuronal development, folic acid fortification is everywhere from elderly diet to antenatal care. It should be noted that B12 deficiency is quite common in pregnancy due to high

demand. In fact, one prospective study has reported that the intakes of low B12 high folate diet in pregnant mothers have high risk of small-for-gestational-age (Dwarkanath *et al* 2013). The possible mechanism could be that the associated dyslipidaemia and high homocysteine levels in vitamin B12 deficient mothers disturb with placenta blood flow, thereby, altering the size of intrauterine baby (Hogeveen *et al* 2012; Adaikalakoteswari *et al* 2015b). Although adequate vitamin B12 levels can help improve these outcomes by providing enough methyl groups for normal foetal programming, there is limited data to support that vitamin B12 supplementation are beneficial. Thus, considering the importance of vitamin B12 in pregnancy, epidemiological and mechanistic studies looking at vitamin B12 deficiency and supplementation are highly demanded before implementation of vitamin B12 at population levels.

In conclusion, vitamin B12 deficiency can impede methylation of lipid synthesis enzymes as well as the efficacy of metformin, particularly its lipid lowering effects, can be impaired in vitamin B12 deficiency. Thus, before metformin is given, it should be considered to check vitamin B12 levels.

CHAPTER 7

THESIS SUMMARY AND CONCLUSIONS

7.1 Overview

Since 1954 when the term GDM has been first developed, the optimal GDM care has not been agreed upon internationally. With different diagnostic criteria and prevalences worldwide ranging from 5-25%, GDM is in the upward trend successively. The target of GDM management is to keep the pregnancy safe from adverse effects of hyperglycemia. Diet and lifestyle intervention is the first strategy to achieve the target glycemic levels and if required, insulin is the universally acceptable medication to add on. For the past few years, metformin role in pregnancy is found to be beneficial in many aspects of GDM management. However, because of placental drug transfer and its long-term concerns, it is not widely used in pregnancy yet. Moreover, the use of metformin is also associated with reduction in serum vitamin B12 and folate levels which are critical micronutrients for foetal development during pregnancy. Folate being part of antenatal program, the deficiency is not common during pregnancy but vitamin B12 deficiency is. It is also well-known that high folate levels can have detrimental effects on health in patients with vitamin B12 deficiency. Thus, it is important to understand how metformin, vitamin B12 and folate interact with each other to ensure the delivery of metformin in GDM effectively and safely.

This thesis aimed to improve the overall management of GDM by evaluating the current evidence of metformin in GDM, examining the inter-relationship of vitamin B12 and folate on pregnancy outcomes in UK GDM population and finally, study the efficacy of metformin in vitamin B12 deficiency condition.

7.2 Systematic review of metformin in GDM

The role of metformin in GDM is increasing over the last few decades. Evidence from a number of recent meta-anlayses have suggested that metformin could be better medication than insulin for GDM. However, all of them included only randomized controlled trials, most of which are of poor quality. The addition of non-randomized trials data in GDM might help to increase the sample sizes and allow sensitivity analysis to generate more precise outcome estimates.

In line with previous reviews, metformin could be better than glibenclamide and insulin in GDM management. Our meta-analyses was complete and included 9 RCTs and 6 NRCTs which revealed that metformin in GDM can benefit less maternal weight gain, pregnancy-induced hypertension, neonatal hypoglycaemia and NICU admission rates in comparison with insulin. Moreover, the NRCTs meta-analyses have suggested possible reduction of LGA, SGA and macrosomia whereas the sensitivity analysis of RCTs suggested reduced neonatal jaundice and decreased birth weight and macrosomia. Most of the limitations of NRCT findings are that the recruitment of moderate hyperglycemic group as well as exclusion of metformin-treated GDM with additional insulin and GDM women with <10% foetal abdominal circumference in metformin arm. This might favour the better improvement of pregnancy outcomes in metformin-treated arm. Out of 15 studies included, only 2 RCTs are of high quality. Thus, future randomized controlled trials should be focused on GDM with moderate hyperglycemia with adequate power to detect the outcomes suggested by NRCTs. It should be noted that sensitivity analysis of RCTs effect estimate suggests increased risk of prematurity with metformin than insulin. Furthermore, the toddler follow-up metformin data from 2 RCTs suggest comparable outcomes with insulin. There is also

a need of follow-up RCTs with complete measurement of adiposity in these children. At the same time, 3 RCTs with glibenclamide have indicated the increased neonatal hypoglycemic risk with glibenclamide. So far, metformin seems to be the best oral antihyperglycemic agent in GDM.

Moreover, we have systematically collected the data on characters of GDM women who are likely to need additional insulin (metformin failure) in later pregnancy. It is suggested that risk factors of GDM like older age, higher BMI and higher glucose levels can be predictors of metformin failure and metformin failure might also relate to certain ethnic groups. Understanding the factors contributing to metformin failure helped to develop a predictive model to identify subgroup of GDM with potential metformin faiure. Thus, using the retrospective clinical data of metformin users in GDM, we have established the predictive tool in our chapter 4.

Based on current available data, metformin is safe to use in GDM and it also can benefit GDM in many aspects such as increased patients' compliance, saved healthcare budgets and better reallocation of healthcare resources.

7.3 Predictors of metformin failure in GDM

Being a drug with less maternal weight gain, metformin could be a very convincing candidate in GDM to reduce the prevalence of childhood obesity and related risks. However, a significant proportion of metformin-treated GDM required additional insulin for optimal glycaemic control. Some of the studies revealed that the doses of insulin needed after metformin failure are higher than those with insulin initiation after dietary failure. This might suggest poor glycaemic control among metformin-failed GDM which in turn increases foetal insulin stimulation. Thus, if a treatment strategy with metformin in GDM is developed, these high-risk mothers could manage with options other than metformin in order to avoid unnecessary risk.

In our systematic review, it have been reported that some of the maternal baseline characters such as age, BMI, fasting glucose at OGTT and high risk ethnicity are associated with higher risks of insulin resistance could predict metformin failure. Our retrospective study of metformin in GDM (Chapter 4) have also shown that older age, higher fasting glucose at OGTT, higher HbA1c at OGTT and earlier gestational age at dietary failure can be determinants of metformin failure. We have also established a diagnostic algorithm that fasting glucose >4.8mmol/L or fasting glucose ≤4.8mmol/L and GA at OGTT $\leq 27^{+5}$ weeks can detect almost 90% of women who are at risk of metformin failure. Moreover, there are almost 4 in 5 chance of metformin failure among women who have been diagnosed GDM either by IADPSG fasting or NICE 2015 fasting criteria. Finally, women with metformin failure are associated with 2 times higher risk of having premature babies.

Thus, we have concluded that metformin can be an effective or even better alternative to insulin in GDM with certain characteristics. However, GDM with very high fasting glucose levels at OGTT are at high chance of needing supplementary insulin if they are initiated with metformin as first-line medication. This could be avoided by limiting metformin to GDM with moderate hyperglycemia. Moreover, the prospectus GDM diagnosed by IADPSG or NICE 2015 fasting glycemia, insulin or other treatment option other than metformin should be given in order to ensure adequate glycaemic control throughout GDM. Since different criteria of metformin failure were presented with readily available information, this might help healthcare personals in rural areas for confident use of metformin in GDM and identify GDM who might need insulin for tertiary referral. This consequently allow better allocation of resources efficiently. Furthermore, the finding that metformin-failed GDM mothers are likely to deliver premature babies should be replicated by future prospective studies with larger sample sizes as this is an observational data and the confidence interval for the association is very large. In addition, in order to exclude GI side-effects of metformin as a trigger factor for prematurity, future studies with extended-release metformin are required. Finally, as these findings are based on retrospective data, the results should be validated with randomized controlled studies before implementing in policy.

In the absence of such data, it can be concluded that metformin might work better for GDM women with postprandial hyperglycaemia or those with moderate severity.

7.4. Role of vitamin B12 and folates in GDM

Micronutrients, important for cellular differentiation, are essential for normal foetal development. During pregnancy, both vitamin B12 and folate levels are necessary to be sufficient to ensure the optimal one-carbon transfer cycle which is critical for proper methylation and normal foetal programming. Folates being part of antenatal program as 400ug to 5 mg supplementation, it is common to encounter pregnancy with high or normal folate levels in their late trimester. On the other hand, despite high prevalence of vitamin B12 deficiency during pregnancy, it is not given routinely to pregnant women yet. Thus, pregnancies with high/normal folates among low vitamin B12 status can be seen in every antenatal clinic. In fact, vitamin B12 deficiency and GDM are highly associated almost half of GDM women have low vitamin B12 levels. There are a number of reports in pregnancy that high folate supplementation (5mg) in vitamin B12 deficient state can have negative impact on pregnancy outcomes including SGA and diabesity. However, the effect of normal folates among vitamin B12 deficient mothers are yet to be examined.

In contrast to the effects of folic acid supplementation on blood glucose levels in type 2 diabetes, we have found that serum folate levels are inversely associated with OGTT and postpartum fasting glucose levels in vitamin B12 deficient GDM women with antenatal 400ug folic acid intake in Chapter 5. There was no association of serum vitamin B12 and folates with neonatal birth sizes in GDM. This might suggest that it is relatively safe to give folic acid of 400ug supplements to GDM women and this should be accompanied with adequate vitamin B12 levels. Moreover, the homocysteine independent effects of folates on fasting glucose levels might also advocate the direct vascular involvement of folates on vitamin B12 sufficient

endothelial cell rather than homocysteine-related vasculopathy, indicating the role of folic acid supplementation in glycemic control among GDM with normal vitamin B12 levels. Furthermore, here was high correlation between folate levels and homocysteine levels but not vitamin B12. At the same time, high homocysteine levels in GDM can increase the prevalence of SGA among GDM which was around 15% in our study. Thus, it is likely that increased doses of folic acid have a possibility to reduce high rates of SGA among GDM. However, considering the adverse risk of high folates among low vitamin B12 GDM population, this should not be the next preferable option. Instead, studies looking at the impact of 400ug folate intakes in low and normal B12 vitamin GDM on pregnancy outcomes should be examined first. Then, only after replication of this observation of increased prevalence of SGA among normal B12 and folate GDM population, increased folate dose should be another step to bring down the SGA prevalences. This is the first study of impact of normal folate levels in low vitamin B12 state and this should be followed by studying the interaction of normal folate-low vitamin B12 effects in different settings including type 2 diabetes. Overall, this chapter highlights the importance of normal vitamin B12 levels for folates to play its role at full potential.

7.5 Vitamin B12 deficiency on Lipid metabolism and Metformin action in Liver

Metformin is a powerful insulin sensitizing agent and it have a capacity to lower down the blood lipid levels. However, one of its drawbacks includes decreasing serum vitamin B12 levels. As vitamin B12 deficiency in diabetes mellitus is not uncommon association, low vitamin B12 state can be worsened by metformin indication. Being an increasingly popular drug in GDM where vitamin B12 sufficiency is important not only for mothers but also for intrauterine babies, it is important to understand how metformin might work in vitamin B12 sufficient state.

In chapter 7, we have proved that by affecting the genes involved in lipid metabolism through abnormal methylation and reduced AMPK phosphorylation, vitamin B12 deficiency can reduce the potency of lipid-lowering effects of metformin in hepatocytes. Moreover, repeated cultivation of these cells in vitamin B12 supplemented state can reverse these effects. This might indicate that for metformin to have full potential on cellular metabolism, it is important to ensure the cells have grown in normal vitaminB12 levels. For women who would receive metformin during pregnancy, they should have normal vitamin B12 levels before metformin start and throughout the metformin exposure. This could be achieved by supplementing vitamin B12 at multifaceted levels, firstly targeting general population to ensure the normal vitamin B12 levels among reproductive women and then additional supplements to those who are in pregnancy.

Although it is found that vitamin B12 supplementation can improve the efficacy of metformin, the duration and timing of supplementation still needs to be established. As most of vitamin B12 deficient GDM could be detected just before metformin

initiation, the question of whether vitamin B12 supplements together with metformin can have similar efficacy to those on metformin with normal vitamin B12 levels are not yet examined. This should be done by conducting randomized controlled trials among GDM with metformin commencement with or without vitamin B12 supplements. Meanwhile, liver having high capacity for vitamin B12 storage and there is no clinical reports of vitamin B12 toxicity due to supplementation, it might be safe enough to give vitamin B12 supplements to those who are sufficient. Thus, it is better to check vitamin B12 levels of all GDM who are going to be offered metformin and vitamin B12 should be supplemented accordingly to enable metformin to act at full efficiency.

7.6 Overall Conclusions

In conclusion, the studies conducted in this thesis presented the new insights for the use of metformin in GDM women and highlighted the importance of normal vitamin B12 levels for overall improvement in GDM management. Evidence is based on contemporary comprehensive systematic review of metformin in GDM, establishment of different strategies to identify GDM sub-group who could potentially have high risk of metformin failure, understanding the inter-relational effects of folate in normal range in the context of vitamin B12 deficiency on pregnancy outcomes and finally, elucidating the mechanism how metformin works in vitamin B12 deficient cell. It is hoped that the findings from these studies will inform the health policy makers in implementing metformin as first-line in GDM guidelines and will facilitate diabetes specialists to provide the best GDM care as possible with metformin.

LISTS OF ABSTRACTS AND PRESENTATIONS

1. **Oral Presentation**

   Khin MO, Vatish M, Gates S, Saravanan P**.** (2013) Evaluation of Metformin in Gestational Diabetes: Systematic Review and Meta-analysis, *Diabetic Medicine*, 30:12. *Diabetes UK Professional Conference*, Manchester, March 2013.

2. **Poster Discussion**

   i) Khin MO, Gates S, Saravanan P. (2014) Metformin in Gestational Diabetes and Fasting Glucose as a Predictor of Treatment Failure. *Diabetologia*, 57:S450. *50th EASD Annual Meeting*, Vienna, September 2014.

   ii) Khin MO, Vatish M, Gates S, Saravanan P**.** (2013) Evaluation of Metformin in Gestational Diabetes: Systematic Review and Meta-analysis, *Diabetologia* 56:S504. *49th EASD Annual Meeting*, Barcelona, September 2013.

3. **Poster Presentation**

   i) Khin MO, Antonysunil A, Kumsaiyai W, Voyias P, Gates S, Mcternan P, Tripathi G, Saravanan P. Molecular mechanism of metformin in Vitamin B12 deficient liver cells. *British Endocrine Society 2015 annual meeting*. Edinburgh, November 2015.

   ii) Khin MO, Gates S, Saravanan P. (2015) Correlations of Serum Folate Levels with Glucose Levels in Gestational Diabetes in relation to Vitamin B12 levels, *75th Annual ADA meeting*, Boston, June 2015.

   iii) Khin MO, Gates S, Saravanan P. (2015) Predictors of prematurity in Metformin-treated Gestational Diabetes Mellitus (GDM). *Diabetic Medicine*. *Diabetes UK Professional Conference*, London, March 2015.

   iv) Khin MO, Gates S, Saravanan P. (2015) Maternal Homocysteine levels at the time of diagnosis independently predicts small-for-gestational-age infants in Gestational Diabetes Mellitus (GDM). *Diabetic Medicine*. *Diabetes UK Professional Conference*, London, March 2015.

   v) Khin MO, Gates S, Saravanan P**.** (2014) Predictors of metformin failure in gestational diabetes, *74th Annual ADA meeting*, San Francisco, June 2014.

   vi) Khin MO, Vatish M, Gates S, Saravanan P**.** (2013) Metformin Reduces Maternal Weight Gain in Gestational Diabetes Mellitus (GDM): A Potential for Positive Programming, *8th DOHaD meeting*, Singapore, November 2013.

APPENDICES

9.1 Systematic Review

9.1.1. Search strategy

MedLine (23/5/15)

1. gestational diabetes.mp. or exp *Diabetes, Gestational/
2. pregnancy$ diabetes.mp. or exp *Diabetes, Gestational
3. (impair* adj5 glucose).mp.
4. (impair* adj5 sugar).mp.
5. (abnormal adj5 glucose).mp.
6. (abnormal adj5 sugar).mp.
7. (glucose adj5 intolerance).mp.
8. hyperglycemia.mp. or exp *Hyperglycemia/
9. (high adj5 glucose).mp.
10. (high adj5 sugar).mp.
11. insulin resistan$.mp.
12. 1 or 2 or 3 or 4 or 5 or 6 or 7 or 8 or 9 or 10 or 11
13. metformin.mp. or exp *Metformin/
14. glucophage.mp. or exp *Metformin/
15. exp *Metformin/ or diabex.mp.
16. exp *Methylguanidine/ or dimethylguanidine.mp.
17. exp *Biguanides/ or exp *Metformin/ or dimethylbiguanid$.mp.
18. glumetza.mp. or exp *Metformin/
19. diaformin.mp.
20. diformin.mp.
21. 13 or 14 or 15 or 16 or 17 or 18 or 19 or 20
22. 11 and 21
23. limit 22 to pregnancy
24. exp *Pregnancy/ or exp *Pregnancy Trimester, Third/ or late pregnancy.mp.
25. second trimester.mp. or exp *Pregnancy Trimester, Second/
26. third trimester.mp. or exp *Pregnancy Trimester, Third/
27. 24 or 25 or 26
28. 22 and 27
29. 23 or 28

[mp=title, abstract, original title, name of substance word, subject heading word, protocol supplementary concept, rare disease supplementary concept, unique identifier]

Embase(23/5/15)

1. gestational diabetes.mp. or exp *pregnancy diabetes mellitus/
2. (impair$ adj2 glucose).mp.
3. (impair$ adj2 sugar).mp.
4. (abnormal adj2 sugar).mp.
5. (abnormal adj2 glucose).mp.
6. (high adj2 glucose).mp.
7. (high adj2 sugar).mp.
8. exp *insulin resistance/ or insulin resistan$.mp.
9. hyperglycemia.mp. or exp *hyperglycemia/
10. (glucose adj2 intolerance).mp.
11. pregnancy$ diabetes.mp. or exp *pregnancy diabetes mellitus/
12. 1 or 2 or 3 or 4 or 5 or 6 or 7 or 8 or 9 or 10 or 11
13. exp *third trimester pregnancy/ or pregnancy.mp. or exp *second trimester pregnancy/ or exp *pregnancy/ or exp *"pregnancy disorders of endocrine origin"/ or exp *pregnancy diabetes mellitus/
14. late pregnancy.mp. or exp *third trimester pregnancy/
15. second trimester.mp. or exp *second trimester pregnancy/
16. third trimester.mp. or exp *third trimester pregnancy/
17. 13 or 14 or 15 or 16
18. 12 and 17
19. exp *metformin embonate/ or exp *metformin/ or metformin.mp. or exp *metformin 4 chlorophenoxyacetate/ or exp *metformin glycinate/
20. glucophage.mp. or exp *metformin/
21. diabex.mp. or exp *metformin/
22. glafornil.mp. or exp *metformin/
23. glisulin.mp.
24. exp *metformin/ or risidon.mp.
25. exp *metformin/ or dianben.mp.
26. dimethylbiguanid$.mp. or exp *biguanide derivative/
27. exp *biguanide/ or dimethylguanylguanidine.mp. or exp *metformin/
28. glumetza.mp. or exp *metformin/
29. fortamet.mp. or exp *metformin/
30. glycomet.mp. or exp *metformin/
31. diaformin.mp. or exp *metformin/
32. diformin.mp. or exp *metformin/
33. bolamyn.mp.
34. riomet.mp. or exp *metformin/
35. 19 or 20 or 21 or 22 or 23 or 24 or 25 or 26 or 27 or 28 or 29 or 30 or 31 or 32 or 33 or 34
36. 18 and 35

[mp=title, abstract, subject headings, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword]

The Cochrane Library(23/5/15)

1. "gestational diabetes"
2. "pregnancy* diabetes"
3. impair* Near/2 glucose
4. impair* near/2 sugar
5. abnormal near/2 glucose
6. abnormal near/2 sugar
7. "insulin resistan*"
8. glucose near/2 intolerance
9. hyperglycemia
10. high near/2 glucose
11. high near/2 sugar
12. (#1 OR #2 OR #3 OR #4 OR #5 OR #6 OR #7 OR #8 OR #9 OR #10 OR #11)
13. pregnancy
14. (late pregnancy)
15. second trimester
16. third trimester
17. (#13 OR #14 OR #15 OR #16)
18. (#12 AND #17)
19. metformin
20. glucophage
21. diabex
22. gliphage
23. glafornil
24. merckfomin
25. risidon
26. dianben
27. dimethylbiguanid*
28. dimethylguanylguanidine
29. glumetza
30. fortamet
31. glycomet
32. diaformin

33. diformin
34. bolamyn
35. riomet
36. (#19 OR #20 OR #21 OR #22 OR #23 OR #24 OR #25 OR #26 OR #27 OR #28 OR #29 OR #30 OR #31 OR #32 OR #33 OR #34 OR #35)
37. (#18 AND #36)

CINAHL(23/5/15)

1. (gestational diabetes)
2. (pregnancy$ diabetes)
3. impair$ NEAR2 glucose
4. impair$ NEAR2 sugar
5. abnormal NEAR2 glucose
6. abnormal NEAR2 sugar
7. high NEAR2 glucose
8. high NEAR2 sugar
9. (insulin resistan$)
10. hyperglycemia
11. glucose NEAR2 intolerance
12. 1 OR 2 OR 3 OR 4 OR 5 OR 6 OR 7 OR 8 OR 9 OR 10 OR 11
13. pregnancy
14. (late pregnancy)
15. (second trimester)
16. (third trimester)
17. 13 OR 14 OR 15 OR 16
18. 12 AND 17
19. metformin
20. glucophage
21. diabex
22. gliphage
23. glafornil
24. merckfomin
25. risidon
26. dianben
27. dimethylguanid*
28. dimethylguaniguanidine
29. glumetza
30. fortamet
31. glycomet
32. diaformin
33. diformin
34. bolamyn
35. riomet
36. metformin
37. 19 OR 20 OR 21 OR 22 OR 23 OR 24 OR 25 OR 26 OR 27 OR 28 OR 29 OR 30 OR 31 OR 32 OR 33 OR 34 OR 35 OR 36
38. 18 AND 37

Web of science(23/5/15)

1. Topic=("gestational diabetes") OR Topic=("pregnancy* diabetes") OR Topic=(impair* NEAR/2 glucose) OR Topic=(impair* NEAR/2 sugar) OR Topic=(abnormal NEAR/2 glucose) OR Topic=(abnormal NEAR/2 sugar) OR Topic=(high NEAR/2 glucose) OR Topic=(high NEAR/2 sugar) OR Topic=("insulin reistan*") OR Topic=(hyperglycemia) OR Topic=(glucose NEAR/2 intolerance)
2. Topic=(pregnancy) OR Topic=("late pregnancy") OR Topic=("second trimester") OR Topic=("third trimester")
3. 2 AND 1
4. Topic=(metformin) OR Topic=(glucophage) OR Topic=(diabex) OR Topic=(gliphage) OR Topic=(glafornil) OR Topic=(merckfomin) OR Topic=(risidon) OR Topic=(diaben) OR Topic=(dimethylbiguanid*) OR Topic=(dimethylguanylguanid*) OR Topic=(glumetza) OR Topic=(fortamet) OR Topic=(glycomet) OR Topic=(diaformin) OR Topic=(diformin) OR Topic=(bolamyn) OR Topic=(riomet)
5. 4 AND 3

9.1.2. Data proforma sheet (systematic review)

Evaluation of Metformin in Gestational Diabetes

Data Extraction Proforma

1. Source

Study ID	
Report ID	
Country	
Language	
Date of study	
Authors, Contact details	
Citation	

2. Eligibility

Population	
Intervention	
Study Design	
Outcomes considered	
Reason for exclusion	

3. Methods

Duration	
Setting	
Sample size	
Total number of participants at baseline	
Total number of participants during analysis	
Intention-to-treat analysis?	

The reason of missing	

Participants

Inclusion and exclusion criteria	
Screening method	
Diagnostic criteria	
Lifestyle intervention	
The level at which drug treatment was started	

Characteristics of Participants

Character	Intervention 1	Intervention 2	Intervention 3	Dif	Note
Age(yr)					
Early pregnancy weight					
Weight at entry					
Weight gain during study					
GA at entry					
GA at OGTT					
Ethnicity					
Glucose level at OGTT					
Glucose level at entry					
Glucose control throughout pregnany					
Smoking n(%)					
Parity/Gravidity n(%)					
Nullip n(%)					
History of chronic hypertension					
BP at enrolment					

Previous history of n(%) GDM PE GHT Macrosomia CS <37 wks Infant with congenital anomaly Bad obstetric history					
No of pregnancy					
Family h/o of diabetes Hypertension preeclampsia					
Foetal abdo circumference %					
Education					

Intervention

Specific intervention	Intervention 1(n)	Intervention 2(n)	
i.duration ii.dose iii.particular drug used iv.Maximum dose v.if fail to achieve target, insulin added/replaced?insulin used(type and dose)? vi.proportion of GDM failed to achieve target with specific intervtn, n(%)			
Integrity of intervention			
Target glucose level			

Design

For randomized study,

Sequence generation	
Allocation sequence concealment	
Blinding(participants, personnel and Outcome assessors)	
Other bias	
Type of analysis	

For observational study,

Was there a comparison?	
Were groups created by?	
Baseline demographic factors comparability	
Statistical methods to control confounding Design Analysis	

Outcomes

Outcome	Intvtn 1	Intvtn 2	Intvtn 3	p	RR	Diff	Note
Foetal BWt in g							
Macrosomia							
LGA							
SGA							
BWt centile							
MOD(%)							

Hypoglycemia							
Cord blood C-peptide/insulin							
SCBU admission Rate & duration							
Perinatal mortality							
GA at birth							
Major or minor abnormality							
Shoulder dystocia							
Birth trauma							
Nerve palsy							
fractures							
Cephalhaematoma							
Respiratory distress							
Cord artery pH							
Neonatal Jaundice(n)							
Additional note							

Secondary
Baby

Outcome	Intervention 1	Intervention 2	Intervention 3	p	RR	Note
APGAR score						
Arthropometric						

Mother

Outcome	Ivt 1	Ivt 2	Ivt 3	p	RR	Note
HCP						
Induction of labour						
Antepartum complication						
Intrapartum complication						
Postpartum complication						
Postnatal GTT						
Postnatal psychological changes						
Hypoglycemic event						
Change in HbA1c						
Cost						
Additional note						

Any subgroup analysis

Any sensitivity analysis

4. Others

Ethical approval	
Funding	
Conflict of interest	

5. Conclusion

Study conclusion	
Reviewer conclusion	
Comment GRADE quality Level of evidence	

User guide for data extraction proforma

Basically, try to collect the data as specific and comprehensive as possible using short terms. For the information that needs for justification of the quality of the study, collect direct quotation with "--".

If you intend to justify the item, use appropriate term for your justification with the referring page number and line numbers where you get such information. E.g. some study may give dietary management in details and if you justify it is appropriate , note down your justification with reference to the text.

Source

Study ID – Last name of the first author, publication year

Report ID – date/mth/year

Country – where the study was conducted

Language –

Date of study – as specific as possible

Authors – maximum 3 authors. Last name with initials of first and middle name

Contact details – contact author full name, address with email(preferable).

Citation –

Eligibility

i)Population –

how was gestational diabetes diagnosed(75 g/100g OGTT)?(name only)

Gestational period during which the test was assumed to be performed.

Proportion of gestational diabetes if the population was mixed with other types of diabetes

ii)Intervention – Is there a separate arm for metformin? Code Y – Yes, N – No.

Which interventions were compared to metformin?(name only)

iii)Design – randomized or observational?(name only)

iv)Outcomes considered – refer to objectives of the study and primary outcomes considered in the method section(if any).

Methods

Duration – in months(mth)

Setting – where the study was conducted(describe all hospitals & clinics involved).

Sample size – Is there any sample size calculation before the study was started?

If yes, describe α value, β value, outcome of interest,

the estimated difference of the outcome considered and the number needed.

Number of participants at the start – collect the number in separate group. 'At the start' refers to the number of participants in each group after randomization in randomized trial where as in non-randomised study, it refers to the numbers of whose the researcher collected for baseline data.

Total number of participants at the end – collect the number in separate group. 'At the end' refers to the number of participants included during analysis.

Intention-to-treat analysis - (Y – yes, N- No)

The reason of missing – e.g. loss of data

<u>Participants</u>

Inclusion and exclusion criteria - all the criteria the participants must be fulfilled so that she would be eligible for screening

Screening(methods and details) – refers to risk factor based or oral glucose challenge test based screening to identify pregnant women to offer diagnostic oral glucose test(OGTT).

Diagnostic criteria –

The level at which was considered to be impaired glucose level and start lifestyle intervention.

Describe the type of OGTT and number & type of blood glucose level needed to be considered impaired.

Lifestyle intervention –

Describe the dietary advice in details(who gave the advice and what was it?).

How the blood glucose was monitored(timing, frequency, device used, duration) with comparable care pathway or not.

The level at which drug treatment was started –

Describe the values of impaired glucose control in details(type, number, etc).

Characteristics of participants table

Row : character

Column : add specific drug name in the intervention box with number of participants on which the analysis of the characters are based;

p : $p<0.05$ – statistically significant difference;

note : describe the number of participants in each group if they differed with baseline numbers, the specific definition of character term(e.g. self-reported in ethnicity row) and other specific consideration for each term.

Definition of the character terms(entry/enrollment = the time at which drug treatment was commenced, n= number)

Age –

Early pregnancy BMI, weight – All BMI or weight value taken before OGTT was given(when the OGTT was done).

BMI at entry – BMI when they were given drug treatment.

Weight change – the increase or decrease of weight from the start of the drug treatment to the late trimester(36/37 weeks gestation)

GA at the start – all gestational age apart from that at enrollment

GA at entry – gestational age(GA) at which drug treatment was started

Ethnicity –

Glucose level at the start – blood glucose level apart from that at enrollment

Glucose level at entry – blood glucose level at which drug treatment was started

(Fasting-FBS, Postprandial- PP, Hb A1c)

Glucose control throughout pregnancy – from the start of the drug treatment to the end of gestation or from diagnosis of OGTT to the end of gestation

(Fasting-FBS, Postprandial- PP, Hb A1c)

Blood pressure(BP) at enrollment

Past history of chronic hypertension

Smoking

Parity/Gravidity – number of previous pregnancy

Nullip – number of woman who never bore a baby

Previous obstetric history of gestational diabetes(GDM), preeclampsia(PE), gestational hypertension(GHT), Macrosomia, Caesarean section(CS), premature baby, infant with congenital anomaly, Bad obstetric history(e.g.recurrent miscarriage(≥3 termination ofpregnancy)), baby with birth defect

Number of pregnancy – number of baby in current pregnancy(single/multiple)

Family history of – diabetes, hypertension, preeclampsia

Foetal abdominal circumference

Education

Intervention

Duration – the time during which the intervention was taken

Dose – average or total dose of the intervention

Particular drug used – brand name, start dose, method of increment over a period

Target glucose level – describe if it was different from the level at which drug treatment was started

Maximum dose – beyond which was considered to be treatment failure for oral hypoglycemic agents

If fail to achieve target, insulin added/replaced? Describe type, dose and duration if any.

Proportion of treatment failure –

Integrity of intervention –

It includes number of participants in each group who stopped taking the treatment before the end of pregnancy apart from those with treatment failure. Describe the number and reasons and what was done(stop treatment or reduce the dose).

Design

Use the table according to the study design(randomized/observational).

For randomized study, record the direct quote from the paper as much as possible. If it is not possible for direct quotation, describe the relevant key information in short term with relevant reference.

For observational study,

Was there a comparison? – respond Y- yes or N-no, number of comparison,

Were groups created by?- describe how each group was formed and if there was any

difference between group formation- time, location, patient preference, etc)

Baseline demographic factors comparability – Any other different character between the 2 groups apart from that was listed in characteristics of participants table

Statistical methods done to control confounding –

Design – matching, stratification, exclusion

Analysis – regression model, sensitivity analysis, stratification

Outcomes

Describe each outcome in definition(how the outcome was assessed) if any in note box and each intervention with number analysed . If the outcomes was not considered or described, put "-" in the box.Add number analysed for particular outcome if it was different.

Primary

Foetal birthweight/Macrosomia/Large for gestational age(LGA)/Small for gestational age(SGA)/BWt centile

Mode of delivery(MOD) – vaginal/normal spontaneous delivery/ instrumental/ assisted/ caesarean section(CS)

Hypoglycemia according to definition given,

Cord blood C peptide/insulin

SCBU admission – SCBU(special care baby unit)/NICU(neonatal intensive care unit)admission rate &/or duration

Perinatal mortality – the number of baby which was not born alive(still birth, miscarriage/abortion, perinatal death)

GA at birth – the value that indicates how mature the baby born(preterm, term, full-term)

Major or minor abnormality – all congenital anomaly

Shoulder dystocia

Birth trauma

Nerve palsy

Fractures

Cephalhaematoma

Respiratory distress

Cord artery pH

Neonatal Jaundice according to the definition (those needing phototherapy or those with hyperbilirubinaemia)

Additional note

Secondary

APGAR score according to the text given(in number with APGAR 7, or specific APGAR score)

Arthropometric measure(at birth)

Mother

Hypertensive complication of pregnancy(HCP)- the increased blood pressure that was first developed during preganancy, gestational hypertension(GHT), preeclampsia(PE), Eclampsia

Induction of labour

Antepartum complication(Polyhydramnios, APH, Abruption, Antepartum infection)

Intrapartum complication(obstructed labour, prolonged labour, instrumental delivery, 3rd degree tear)

Postpartum complication(postpartum/peripartum haemorrhage, postpartum infection)

Postnatal GTT – oral glucose test result (number or any glucose valuethat was taken after the delivery of baby)

Postnatal psychological changes

Hypoglycemic event

Change in Hb A1c

Cost -

Additional note

Subgroup analysis, e.g. Metformin alone vs metformin failure

Sensitivity analysis, e.g. the results of the outcomes after confounders were being controlled.

Others

Ethical approval –

Funding body

Conflict of interest

9.1.3. Risk of bias tools

9.3.1 Risk of bias tool(RCT)

Items	(page, lines)	Justification	Code
1.Randomization			
2.Allocation concealment			
3. Double Blinding			
4.Same Care Pathway			
5.Blinding of outcome assessors			
6.Prior sample size			
7.Intention to treat analysis			
8.Acceptable attrition rate			
9.Comprehensive outcome report			
10. Free from other risk of bias			
11.Population			
12.Intervention			
13. Comparator			
14.Explicit reported outcomes			

User guide for risk of bias tool for randomized studies

Coding instruction

'+ 'means 'yes' to the question item that the item is stated in the report; information given is adequate enough to justify that there is low risk of bias.
'- ' means 'no' to the question item that information given is adequate enough to justify high risk of bias or the item is not stated in the report
'?' means'unclear' to the question item that the item is stated but information given is not adequate to justify the risk of bias.

General items

1. Randomization

Did the study describe proper randomization method?+, -, ?

At the start of the study, the researcher needs to ensure that the chance of all participants to be involved in each intervention group must be equal. The justification was based on the method used to allocate participants to each treatment group, taking account of the fact that it is truly random. E.g of adequate randomization includes using a computer random number generator, referring to a random number table. E.g. of inadequate randomization includes sequence generated by odd or even date of birth, some rule based on hospital or clinic record number.

2. Allocation concealment

Did the study adequately prevent assignment of participants to people involved in the research?+,-,?

The sequence of the enrolling participant assigned to the intervention group must be adequately concealed, i.e, participants, investigators and caregivers could not foresee who was assigned to which group. E.g. of appropriate concealment includes sequentially numbered, opaque, sealed envelopes; central allocation. E.g. of inappropriate concealment includes alternation; open random allocation schedule.

3. Double blinding

Did the study adequately blind who received which treatment from participants and researchers?

The double blinding of the study allows unbiased attention and care of the participants independent of treatment type. It should be noted that if the routes of intervention are different (subcutaneous injection route in insulin and oral route in metformin), it is difficult to blind the participants from the intervention. If the study claimed to be double-blinded in this case, the details on how double-blinding was performed should be described.

4. Same carepathway

Did every included participants get enough and equal attention and care regardless of the intervention? +/-/?

For a prospective study, both subjects in 2 intervention arms should be managed by the same care pathway, same team at the same setting over the same follow-up frequency and period.

5. Blinding of outcome assessors (including those involved in data analysis) +,- , ?

Were the outcome assessors adequately blinded to the intervention the participants assigned?

In trials comparing different routes of administration or different sizes of drugs where it might be difficult to be properly blinded to the caregivers and participants, it is crucial to ensure the adequate blinding of outcome assessors. The justification for this item is "No" if either outcome assessor or data analyst is not properly blinded or information is not given.

6. Prior sample size

Did the study describe proper sample size calculation? +, -, ?

Before any study is conducted, the number of participants required in each group to detect the difference in outcome of interest should be adequately calculated and the study should recruit enough number of participants to minimize the play of chance.

7. Intention to treat analysis

Were outcomes of all participants randomized measured and included in the analysis as initially randomized group? +,-

A response of "Yes" to each part of the above question is justified to be low risk of bias. Alternative is true to high risk of bias.

8. Acceptable attrition rate +, -

If there is missing data, were they equal in terms of number and reason in each arm?
Was the attrition rate not enough to have impact on the effects of intervention?

The attrition rate of <20% is considered to be acceptable if the missing data were equal in terms of number and reasons.
To have a record of "+", the response to both question items must be YES.

9. Comprehensive outcome report

Was the study free from selective outcome reporting at study-level judgement?

In practice, it is difficult to assess the selective outcome reporting. But, it is suggested that at least all outcomes that the researcher mentioned in the method section needed to be reported in results. If the study failed to report the outcomes reported by most of the studies, it could be suspicious that the study might be intentionally omit the outcomes. Moreover, if the study did not report the related outcomes together(large for gestational age and low for gestational age), risk of selective reporting bias should be suspected. Furthermore, if the study failed to report any of important clinical outcomes considered in this review, "-" sign should be noted.

10. Other risks of bias

Was the study free from other potential risks of bias? State them.

Other quality assessment items which could introduce bias to the quality of the study includes early stopping, baseline imbalance, blocked randomization in unblinded trials and interim results. In unblinded study, block randomization could introduce selection bias as it is probable that the next allocated intervention could be predicted. The level of type I error increases with the number of interim analyses("if the investigator looks at the accumulating data at α=0.05 at every interim, then the actual overall α level rises with the number of challenges – eg, overall α=0.08 after 2 challenges, α=0.11 after three and α=0.19 after ten.[250]")

11. Population

Did the study clearly describe the eligible subjects, those included and excluded?+/-/?

12. Intervention

Did the study clearly report explicit intervention used?+/-/?

The study should report the details of the intervention(start dose, frequency, maximum dose, how to increase or decrease the dose, time awaited to justify inadequate intervention). It is important either for generalizability of the study or the reasons of difference in proportion of participants who suffered from treatment side-effects.

13. Comparator

Did the study clearly report explicit comparator(e.g.insulin) used?+/-/?

The study should report the details of the intervention(start dose, type, frequency, maximum dose, how to increase or decrease the dose, time awaited to justify inadequate intervention). It is important either for generalizability of the study or the reasons of difference in proportion of participants who suffered from treatment side-effects.

14. Explicit outcome +/-/?

Were the reported outcomes clearly defined?

Some clinical outcomes(respiratory distress, neonatal hyperbilirubinaemia or neonatal jaundice, neonatal hypoglycemia) were necessary to be clearly defined in order to ensure the comparability across studies and generalizability.

Specific items

9.3.2 Risk of bias tool (NRCT)

Items	(page, lines)	Justification	Code
1.Consecutive sample			
2. Unbiased Selection criteria			
3.Baseline comparability			
4.Same care pathway			
5.Favours group			
6.Blinding of outcome assessors			
7.Prior sample size			
8.Intention-to-treat analysis			
9.Complete outcome report			
10. Confounders controlled			
11.Population			
12.Intervention			
13. Comparator			
14.Explicit outcomes			

User guide for risk of bias tool for observational studies

General Coding instruction

'+ 'means 'yes' to the question item that the item is stated in the report; information given is adequate enough to justify that there is low risk of bias
'- ' means 'no' to the question item that information given is adequate enough to justify high risk of bias or the item is not stated in the report
'?' means'unclear' to the question item that the item is stated but information given is not adequate to justify the risk of bias

General items

1. Consecutive sample

Were the participants recruited as a consecutive sample?+/-/?

In observational study, it is difficult to assess the selection bias. In order to avoid bias in selection of patients, one might ensure that all the participants who are eligible and accessible to the study should be recruited and included in data analysis. Accordingly, all potentially eligible subjects for the study should be clearly defined in terms of place and time period as well as selection criteria for those selected for intervention arm and comparator arm. If there is any reason to leave out a particular group of patients, the reason must have been recorded. For example, if the study is intended to recruit all GDM in between 2009-2010 at a particular hospital, data from all GDM mothers who bore her baby during that period are considered to be eligible population.

2. Unbiased selection criteria

How the group was formed?

This item aims to assess whether the health care professionals or the personnels who are responsible to patients recruitment could foresee and could determine the patients' allocation.
If patients are allocated according to site in a prospective cohort study, it is justified that the responsible person for patients' recruitment could foresee the outcomes or might have some biases in his decision and there is probable high risk of bias. If the allocation depends on patients' choice, it is justifiable that it is unlikely to foresee the outcome and there might be low risk of biases.

3. Baseline comparability

Were there any significant difference(clinical or statistical) in between group?
What are they?

4. Same carepathway

Did every included participants get enough and equal attention and care regardless of the intervention? +/-/?

For a prospective study, both subjects in 2 intervention arms should be managed by the same care pathway, same team at the same setting over the same follow-up frequency and period.

5. Favours group

Which intervention group have less glycaemic risk factors at baseline?
The box should be filled in with abbreviation of intervention(D=Diet, M=Metformin, I=Insulin, G=Glibenclamide, MF=Metformin failure)

The favourable group in this item means the group with less insulin resistance states (e.g. younger age, lower BMI, lower baseline glucose, etc).

6. Blinding of outcome assessors (including those involved in data analysis) +,- , ?

Were the outcome assessors adequately blinded to the intervention the participants assigned?

Although blinding is not a common practice in observational studies, in trials comparing different routes of administration or different sizes of drugs where it might be difficult to be properly blinded to the caregivers and participants, it is crucial to ensure the adequate blinding of outcome assessors. The justification for this item is “No” if either outcome assessor or data analyst is not properly blinded or information is not given.

7. Prior sample size

Did the study describe proper sample size calculation? +, -, ?

Before any study is conducted, the number of participants required in each group to detect the difference in outcome of interest should be adequately calculated and the study should recruit enough number of participants to minimize the play of chance.

8. Intention-to-treat analysis

Was the study justified that the attrition rate is not biased enough to have an impact on the outcome? -/ ?/ +/ ++

All the consecutive population should be included in the analysis section. If there is missing data, the data should be balanced between 2 intervention arms(+) and the attrition rate is not more than 20%(+). If the information is not clear enough to justify the consecutive population, the sign”?” should be noted.

9. Complete outcome report

Was the study free from selective outcome reporting at study-level judgement?

In practice, it is difficult to assess the selective outcome reporting. But, it is suggested that at least all outcomes that the researcher mentioned in the method section needed to be reported in results. If the study failed to report the outcomes reported by most of the studies, it could be suspicious that the study might be intentionally omit the outcomes. Moreover, if the study did not report the related outcomes together(large for gestational age and low for gestational age), risk of selective reporting bias should be suspected. Furthermore, if the study failed to report any of important clinical outcomes considered in this review, “-“ sign should be noted.

10. Confounders controlled

Did the investigator consider justify enough confounders in design and analysis sections.
Tips : list and compare confounders in all studies.
The major confounders considered for this review included age, parity, prepregnancy BMI, weight gain during pregnancy, blood glucose at OGTT, hypertension history, Bad obstetric history(hypertensive complications of pregnancy, LGA, Stillbirth, cesarean section).

Please refer to the confounder table. (Appendix 9.1.5)

(-) the investigator did not consider any confounder or did not attempt to control any confounder in design and analysis section.
(+) the investigator considered some major confounders to the primary outcome and tried to control them.
(++) the investigator considered all major confounders to the primary outcome and tried to control all of them.
(+++) the investigator considered and controlled enough major confounders and we are confident that the primary outcome result could not be influenced by confounders.

11. Population

Did the study clearly describe the eligible subjects, those included and excluded?

12. Intervention

Did the study clearly report explicit intervention used?

The study should report the details of the intervention(start dose, frequency, maximum dose, how to increase or decrease the dose, time awaited to justify inadequate intervention). It is important either for generalizability of the study or the reasons of difference in proportion of participants who suffered from treatment side-effects.

13. Comparator

Did the study clearly report explicit comparator(e.g.insulin) used?+/-/?

The study should report the details of the intervention(start dose, type, frequency, maximum dose, how to increase or decrease the dose, time awaited to justify inadequate intervention). It is important either for generalizability of the study or the reasons of difference in proportion of participants who suffered from treatment side-effects

14. Explicit outcomes +/-/?

Were outcomes clearly defined?

Some clinical outcomes(respiratory distress, neonatal hyperbilirubinaemia or neonatal jaundice, neonatal hypoglycemia) were necessary to be clearly defined in order to ensure the comparability across studies and generalizability.

9.1.4. Description of potentially relevant conference proceedings and ongoing trials for systematic review

Table 9.1. Description of RCTs studies (Metformin vs Insulin)

Study ID	Design	Participants	Intervention	Outcomes
Rnnemaa 2010[251]	Prospective cohort study of ongoing randomized trial	150 GDM pregnancies; FBS > 7 mmol/L or PP > 11 mmol/L were not included in this trial. 75 in metformin vs 75 in insulin group Mean maternal age (31.8 vs 32.2 years), pre-gestational BMI (29.3 vs 39.5 wks), glucose values in pre-treatment OGTT or gestational weeks at delivery (39.3 vs 39.5) did not differ between the two treatment groups.	Metformin insulin Criteria for impaired glucose control or when to add additional insulin : FBS >5.5mmol/l and/or PP >7.8mmol/L	Outcomes compared: Metformin vs insulin in GDM mothers stratified by pre-gestational BMI 30 kg/m2 into obese and non-obese. Perinatal outcomes 1. Birth weight(g, mean)- higher in pre-gestationally obese compared with non-obese mothers(3658g vs 3556 g, p=0.19) - Similar in metformin vs insulin group irrespective of maternal pre-gestational BMI. 2. Macrosomia (low in all study group) 3. Metformin failure rate (20%)
Martinez 2010[252]	Randomized prospective trial	100 first GDM patients(final number 278) requiring pharmacological therapy Maternal demographic or obstetric data did not differ between the groups	Metformin Insulin	Metformin vs insulin group Perinatal outcomes: No significant difference in neonatal hypoglycaemia or birth weight percentile, NICU transmission, 5 min APGAR scores and umbilical artery pH. Significant differences ($p<0.05$) were found in neonatal breathing problem (more in insulin group) and vacuum assisted deliveries (more in insulin group). Metformin failure rate (20%)
Bell2010[253]	Cohort study Setting : five maternity units in north-east England	116 GDM delivered between April 2008 – June 2009 screened by blood glucose measurement at booking and 28 weeks but data were available for 103 women(52% obese; 16% non-white ethnicity; 30% family history of diabetes; 12% previous GDM history; 24% no risk factors defined by national guidance; 20% diagnosed before 24 weeks).	34% GDM dietary advice only; 38% insulin alone; 6% metformin alone; 13% both metformin and insulin during pregnancy	Infant outcomes: 105 births (one stillbirth, three major congenital anomaly, two > 4500g birth weight. Maternal outcomes: 49(48%) delivered by caesarean section; 14% at ≥ 39 weeks gestation; 59% had postnatal OGTT and further 13% had fasting glucose only – two diabetes women and six (10%) impaired glucose tolerance.

Table 9.1. Description of RCTs studies (Metformin vs Insulin)

Study ID	Design	Participants	Intervention	Outcomes
Hayden 2010[254]	Retrospective case note review(audit) Setting : Heartlands hospital	17 GDM women treated with metformin	Metformin vs standards(insulin) for pregnancy outcomes set by NICE guidelines 2008	Perinatal outcomes : 1. Foetal macrosomia (n=1, 7%) 2.NICU admission (n=1) 3.No neonatal hypoglycaemia 4.gestational age at birth(64.2% of neonates were born between 38 and 39 weeks gestation, 1 neonate was born under 38 weeks(37+0), 4 born over 39 weeks) Maternal outcomes : 1. Emergency caesarean rate (15.4%) but did not exceed the background population (15.5%) (Both due to failure to progress following induction between 38 - 39 weeks). 2. optimal glucose control in 80% of the GDM
Hashmi 2012[255]	A retrospective cohort study Setting: Pakistan (Jan 2009 to June 2010)	110 GDM(53 metformin-treated and 56 insulin-treated)	Metformin vs insulin	-Similar birth weight(2.9 kg in metformin(M) group vs 2.88 kg in insulin(I) group) -comparable SGA; 5.7%(M) vs 9.3%(I) -higher gestational hypertension in insulin group; 13.7%(M) vs 22.2%(I), p=0.31 -one neonate with respiratory distress syndrome vs none in insulin group - similar NICU admission rate; 7.8%(M) vs 3.6%(I), p=0.42

9.2. Characteristics of relevant conference proceedings (Metformin vs Glibenclamide)

Study ID	Design	Participants	Intervention	Outcomes
Bertini 2011[256]	RCT July 2008 to September 2010	200 GDM women who required adjunctive (metformin) therapy to diet and insulin therapy. 104 GDM women in metformin group vs 96 GDM women in glibenclamide group No difference was found(p>0.05) in the groups regarding maternal age, gestational age at inclusion, body mass index, glucose levels in 75g OGTT and glycemic control. However, higher numbers of previous pregnancies in Metformin group(2.84 vs 2.47, p=0.04) less weight gain during pregnancy in Metformin group (7.78 vs 9.84 p=0.04)	Metformin Glibenclamide Both drugs were replaced by insulin when they reached the maximum dose without glycemic control.	Metformin(M) vsGlibenclamide(G) group (assessed outcomes : weight and neonatal blood glucose) Perinatal outcomes: No differences (p>0.05) in caesarean delivery, gestational age at delivery, number of LGA newborns, neonatal hypoglycaemia, admission to intensive care unit and perinatal death. Differences were found in 1. Birth weight(g): 3193 g(M)vs 3387 g(G), p=0.01 2. Ponderal index: 2.87(M) vs 2.96(G), p=0.05 Neonatal blood glucose levels in 1st (59.78(M) vs 54.08(G), p=0.01) and 3rd hour (61.53(M) vs 55.89(G), p=0.01)

Table 9.3. Characteristics of relevant conference proceedings (Metformin vs Diet)

Study ID	Design	Participants	Intervention	Outcomes
Gururaj 2010[257]	Retrospective study (?)	46 GDM (January 2008 to June 2009) form pregnancy database; 46 GDM identified of which 28 were treated with metformin. Age, BMI and parity were similar.	(28)Metformin vs (18) Diet only	Glucose tolerance test values at diagnosis were higher in metformin compared to diet (4.91±0.6 vs 4.63±0.3). Significant reduction in 2hr post GTT values at 6 weeks postpartum in both groups (metformin: 5.76±1.7, p<0.0002 and diet: 4.78±0.9, p<0.002 from baseline) and fasting glucose level fall by -0.14% in metformin and rose by 0.43% in diet group.

Table 9.4. Characteristics of relevant conference proceedings (Metformin monotherapy vs Metformin Failure)

Study ID	Design	Participants	Intervention	Outcomes
Siddaramaiah 2011[258]	Retrospective audit	66 GDM women with singleton pregnancies Booking BMI : 31.3(7.8) kg/m2, 49.1% of women had BMI ≥ 30 kg/m2	Metformin: Mean duration 8.3(6.9) weeks; six (9.2%) had intolerance with three switch to insulin; 36.4% required additional insulin	Neonatal outcomes: 7 premature babies (10.7%), mean birth weight (3.44 kg, 0.6%), 16.9% were over 97 percentile, 7(10.7%) NICU admission due to prematurity or hypoglycaemia, 4(6.1%) transiently APGAR < 7 at 1 min, 23% hypoglycaemia(<2.6% mmol/l) ,46 % of whose mothers received insulin, no perinatal death Maternal outcomes : mean weight gain 9.24 kg (from booking to GDM diagnosis) and 1.44 kg (from GDM diagnosis to term); 1.5% pregnancy related complications(PIH, eclampsia, hyper emesis and miscarriage); 3% PET, Ante partum haemorrhage, polyhydramnios,
Bertini 2011[259]	Retrospective study of RCT	104 GDM women who required adjunctive (metformin) therapy to diet and physical activity. 82 GDM women in success group vs 22 GDM women in failure group	Criteria for metformin failure or when to add insulin : absence of glycemic control obtained or foetal abdominal circumference ≥90%	Epidemiological characteristics of the two groups were compared. Maternal age (32.42 vs 32.38, p=0.96), parity (2.73 vs 3.19, p=0.13), weight gain during pregnancy (7.30 vs 5.38, p= 0.13), 2hr OGTT (164.15 vs 167.15, p=0.56) were comparable between the two groups (p>0.05). However, later gestational age at inclusion (27.57 vs 24.33, p=0.04), lower baseline BMI (27.64 vs 31.62, p<0.01) and lower FBS in OGTT l (91.56 vs 108.09, p<0.01) were found in metformin success group.

Table 9.5. Characteristics of ongoing trials

1. JacekBrazert 2008

Study name	Metformin in gestational diabetes mellitus
Methods	Prospective randomized open labelled trial
Participants	18 years of minimum age, inclusion : diabetes diagnosed during pregnancy, single pregnancy, ineffective diet therapy, Exclusion : pre-gestational diabetes, foetal malformation, multiple pregnancy, contraindication to metformin therapy(liver or kidney disease) Setting : Poland
Intervention	Metformin vs human recombined insulin
Outcomes	Primary : newborn weight(1hour of life) Secondary : parameters of metabolic control in mother and newborns : insulin resistance, inflammatory reaction, oxidative stress, foetal growth, during pregnancy and up to 12 hours after delivery
Starting date	May 2008
Contact information	Hanna Mitkowska Wozniak, ph : +48 601 88 60 00 hanna.mitkowska@onet.eu

2. Ware Branch 2011

Study name	
Methods	Prospective randomized, double-blind placebo controlled trial
Participants	100 samples for pilot study Inclusion : Women delivered > 37 weeks gestation baby within last 18 months with history of GDM in immediately prior pregnancy, previous infant > 4000 gms, BMI > 30 6mths postpartum, Hb A1c>6.1% 6 months postpartum, family history of type 2 diabetes, Exclusion : >1 miscarriage or foetal demise, No contraindication to metformin, Hypertension(>135/85), No other endocrine, metabolic, renal or autoimmune medical disorders, history of preterm delivery, neonatal palsy, shoulder dystocia, uterine malformations, illicit drug or alcohol abuse during current pregnancy, multifoetal pregnancy including first trimester embryonic demise of one or more, intent to deliver elsewhere, non-availability for prospective data Setting : Intermountain Healthcare Urban Central region, Utah, United States
Intervention	Metformin vs placebo
Outcomes	Primary - incidence of GDM (pre-conception to delivery)(at 8-week interval) Secondary - maternal and foetal pregnancy outcomes (pre-conception to discharge) (4-week interval)
Starting date	April 2010
Contact information	Camille Broadwater-Hollified, ph : 801-507-7864 camille.hollifield@imail.org

3. Maslovitz 2012

Aim/Hypothesis	To assess whether the addition of metformin to insulin treated pregnant women would improve glucose control and lower insulin requirements
Methods	Retrospective cohort
Participants	64 pregnant women with diabetes requiring > 100 units of insulin. Unbalanced glucose levels requiring further treatment were divided into (40) group A (increasing insulin doses alone) vs (24) Group B (addition of metformin 850 mg bd along with insulin). Two groups were similar in terms of maternal age, body mass index, uterine scar and percentage of pre-gestational diabetes.
Intervention	Metformin vs Insulin
Outcomes	Mean daily insulin : 1352.3 units(A) vs 1053.6 units(B)($p \leq 0.05$) Optimal glycemic control: 8.31.2 days vs 10.11.2 days ($p \leq 0.05$); achieved earlier with combined treatment. Maternal satisfaction score: 4.3 vs 3.6($p \leq 0.05$); better tolerated with combined treatment. Similar birth weight(4385212 g vs 3387138 g), NICU admission rates, neonatal hypoglycemia and macrosomia
Reason for exclusion	Conference abstract, Unclear GDM population
Contact information	S.Maslovitz Department of obstetrics and gynaecology, Lis Maternity Hospital, Isreal. maslovitz@gmail.com

Table Proforma: Assessment of confounders in NRCT

Study ID																				
Confounder	Restriction	Balance	Matching	Analysis	Restriction	Balance	Matching	Analysis	Restriction	Balance	Matching	Analysis	Restriction	Balance	Matching	Analysis	Restriction	Balance	Matching	Analysis
Age																				
Early pregnancy BMI																				
BMI at entry																				
Weight change during study																				
GA at entry																				
GA at OGTT																				
Ethnicity																				
BS at OGTT																				
BS at entry																				
Glucose control throughout pregnancy																				
Smoking																				
Parity																				
Nullip																				
History of chronic hypertension																				
BP at enrollment																				
History of diabetes																				

Study ID																				
Confounder	Restriction	Balance	Matching	Analysis	Restriction	Balance	Matching	Analysis	Restriction	Balance	Matching	Analysis	Restriction	Balance	Matching	Analysis	Restriction	Balance	Matching	Analysis
Previous history -GDM -PE -GHT -Macrosomia -CS -Prematurity -infant with congenital anomaly -bad obstetric history																				
No of pregnancy																				
Family history -diabetes -hypertension -preeclampsia																				
Foetal abdominal circumference																				
Tertiary education																				

NA – No analysis found although the confounder was reported.

? – there is no statistically significant difference in the confounder reported but the reviewer identify the difference as clinical significance

9.2.Clinical Studies

9.2.1 Manual Data proforma sheet for data collection

1. Patient Identification

Hospital no	
Ethnic	
Ethnic of parter	
Religion	
Employment (pt)	
Employment(parter)	
Parter age	

2. Any (+)ve Social History

Smoking details	
Alcohol	
Drug	

3. PMH

Hypertension	
Gestational Diabetes	
PIH	
Any +ve	
Family	
Partner family	

4. Folic acid
   Start date and Dose

5. P/E (gestational wks included)

Wt/BMI at booking	
Wt/BMI at OGTT	
Wt near term	

6. Currently taking drugs (apart from anti-diabetics)

Type	Dose

7. Past obstetric h/o

1st Baby	Alive/SB	
	Sex	
	BWt	
	GA	
	MOD	
	Any C’	
	DOB	
2nd Baby	Alive/SB	
	Sex	
	BWt	
	GA	
	MOD	
	Any C’	
	DOB	
3rd Baby	Alive/SB	
	Sex	
	BWt	
	GA	
	MOD	
	Any C’	
	DOB	
misscarriage	12/40	

8. Current pregnancy

LMP	
EDD based on dating scan	
G, P	
BP at booking	
Gestation at booking	
No of antenatal visit	

9. MOD

Type	
Any indication	
PIH/PE/E	
Any antenatal C’	
Any intrapartum C’	
Any postpartum C’	
Type of Perineal tear	

10. Baby

Alive/SB	
Gest Maturity(wks)	
Sex	
BWt	
Head Circum(cm)	
APGAR	
Cord pH(At)	
Cord pH(V)	
NICU admission (duration, indication)	
Respiratory distress	
Phototherapy/Bilirubin	
DOB	
Glucose after delivery	

11. Glycemic control

Start date	
End date	
>7.8 PP	
> 6 FBS	
<3 any time	
Forget	

9.3.Laboratory Study

9.3.1. Solutions and Buffers

General Cell Culture Solution

1. Lysis Buffer

Ammonium Chloride (NH_4Cl)
Potassium Bicarbonate ($KHCO_3$)

2. Completed MEM media

MEM media (21430-020, Gibco)	500mL
Foetal Bovine Serum	50mL
Pen/Strep	5mL
Pyruvate	5mL

Medium was kept at 4 °C

Western Blotting Solution

1. Sodium Dodecyl Sulphate (SDS) (4%)

SDS	4g
H_2O	100mL

2. Loading Buffer

125 mMTris-HCl (pH 6.8)	625µL
4%SDS	500µL
Glycerol	1mL
Dithiothreitol (DTT)	200µL
Bromophenol Blue	125 µL
Distilled H_2O	250 µL

3. Phosphate Buffer Saline (PBS) (pH 7.6)

Na2HPO4	1.44g
KH2PO4	0.24g
NaCl	8g
KCl	0.2g
Distilled H2O	1L

4. PBS-T

PBS	1L
Tween 20	1mL

5. Blocking solution (20%)

i-Block powder 0.2g
PBS-T 100mL

Solutions used for RT-PCR

1. DNase Treatment

DNase I	Reaction Buffer	Stop Solution
1 U/µL in 50% Glycerol 10mM Tris-HCl (pH 7.5) 10mM $CaCl_2$ 10mM $MgCl_2$	200mM Tris-HCl (pH 8.3) 20mM $MgCl_2$	50mM EDTA

2. Reverse Transcription Buffer

100 mM Tris-HCl (pH 9.0 at 25 °C)
500 mM KCl
1% Triton® X-100

REFERENCES

Adaikalakoteswari, A., Finer, S., Voyias, P. D., McCarthy, C. M.,Vatish, M., Moore, J., Smart-Halajko, M., Bawazeer, N., Al-Daghri, N. M., McTernan, P.G., Kumar, S., Hitman, G.A., Saravanan, P., and Tripathi, G. (2015) Vitamin B12 insufficiency induces cholesterol biosynthesis by limiting s-adenosylmethionine and modulating the methylation of SREBF1 and LDLR genes. *Clin Epigenetics*, 7(1): 1-14.

Adaikalakoteswari, A., Jayashri, R., Sukumar, N., Venkataraman, H., Pradeepa, R., Gokulakrishnan, K., Anjana, R.M., McTernan, P.G., Tripathi, G., Patel, V., Kumar, S., Mohan, V. and Saravanan, P. (2014) Vitamin B12 deficiency is associated with adverse lipid profile in Europeans and Indians with type 2 diabetes. *Cardiovasc Diabetol*, 13: 129;1-7.

Adaikalakoteswari, A., Vatish, M., Lawson, A., Wood, C., Sivakumar, K., McTernan, P.G., Webster, C., Anderson, N., Yajnik, C.S., Tripathi, G., and Saravanan, P. (2015) Low Maternal Vitamin B12 Status Is Associated with Lower Cord Blood HDL Cholesterol in White Caucasians Living in the UK. *Nutrients*, 7(4): 2401-2414.

Adeli, K., Taghibiglou, C., Van Iderstine, S.C., Lewis, G.F. (2001) Mechanisms of hepatic very low-density lipoprotein overproduction in insulin resistance. *Trends Cardiovasc Med*, 11(5): 170-176.

Adiels, M., Taskinen, M.R. and Boren, J. (2008) Fatty liver, insulin resistance, and dyslipidemia. *Curr Diab Rep*, 8(1): 60-64.

Agarwal, M. (2010) Evolution of Screening and Diagnostic Criteria for GDM worldwide. In *Gestational Diabetes During and After Pregnancy* Kim C & Ferrara A, Eds. London, Springer, 35-49.

Alberti, K.G., Zimmet, P.Z. (1998) Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabetic Medicine,*15:539-553

Alwan, N., Tuffnell, D.J., West, J. (2009) Treatments for gestational diabetes. *Cochrane Database of Systematic Reviews,*(3).

American Diabetes Association (ADA) Position Statement. (2004) Gestational diabetes mellitus. *Diabetes Care,* 27:S88-S90

Amin, M., Suksomboon, N., Poolsup, N. and Malik, O. (2015) Comparison of glyburide with metformin in treating gestational diabetes mellitus: a systematic review and meta-analysis. *Clin Drug Investig,*35(6): 343-351.

Andres, E., Loukili, N.H., Noel, E.,Kaltenbach, G., Abdelgheni, M.B., Perrin, A.E., Noblet-Dick, M., Maloisel, F., Schlienger, J.L. and Blickle, J.F. (2004) Vitamin B12 (cobalamin) deficiency in elderly patients. *CMAJ,* 171(3): 251-259.

Ardilouze, J. L., Mahdavian, M. and Baillargeon, J.P. (2010). Brick by brick: Metformin for gestational diabetes mellitus? *Expert Review of Endocrinology and Metabolism,* 5(3): 353-357.

Armstead, E. E., Hsu, J.M. and Chow, B.F. (1971) The incorporation of acetate-2-14C and mevalonate-2-14C into cholesterol during vitamin B12 deficiency. *Proc Soc Exp Biol Med,* 136(3): 911-915.

Bailey, C.J. and Howlett, H.C.S. (2007a) Tolerability of metformin. In *Metformin: The Gold Standard A Scientific Handbook.* Bailey, C.J., Campbell, I.W., Chan, J.C.L., *et al*, Eds. UK, Wiley.

Bailey, C.J. and Howlett, H.C.S. (2007c) Defining the risk of lactic acidosis. In *Metformin: The Gold Standard A Scientific Handbook.* Bailey, C.J., Campbell, I.W., Chan, J.C.L., *et al*, Eds. UK, Wiley.

Bailey, C.J. and Howlett, H.C.S. (2007b) Measures to improve the gastrointestinal tolerability of metformin. In *Metformin: The Gold Standard A Scientific Handbook*. Bailey, C.J., Campbell, I.W., Chan, J.C.L., *et al*, Eds. UK, Wiley.

Bailey, L.B. and Gregory, J.F. (1999) Folate metabolism and requirements. *J Nutr*.129(4): 779-782.

Balani, J., Hyer, S., Rodin, A. and Shehata, H. (2011) Pregnancy outcomes in women with gestational diabetes treated with metformin or di*et al*one. *Obstetric Medicine,* 5: 78-84.

Balani, J., Hyer, S., Rodin, A. and Shehata, H. (2009) Pregnancy outcomes in women with gestational diabetes treated with metformin or insulin: a case-control study. *Diabetic Medicine* 26(8): 798-802.

Ball, G. F. M. (2004a). Folate. *Vitamins: their role in the human body.* London, UK., Blackwell science: 347-377.

Ball, G. F. M. (2004b). Vitamin B12. *Vitamins: their role in the human body.* London, UK., Blackwell Science: 383-392.

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

Baltimore, M. (1999). *Manual of Lipid Disorders: Reducing the Risk for Coronary Heart Disease.*

Barnes, R. A., Edghill, N., Mackenzie, J., Holters, G., Ross, G.P., Jalaludin, B.B. and Flack, J.R. (2013) Predictors of large and small for gestational age birthweight in offspring of women with gestational diabetes mellitus. *Diabet Med*,30(9): 1040-1046.
Barnett, A.H., Dixon, A.N., Bellary, S., Hanif, M.W., O'Hare, J.P., Raymond, N.T. and Kumar, S. (2006) Type 2 diabetes and cardiovascular risk in the UK south Asian community. *Diabetologia*,49: 2234-2246.

Battin, M.R., Obolonkin, V., Rush, E., Hague, W., Coat, S. and Rowan, J. (2015) Blood pressure measurement at two years in offspring of women randomized to a trial of metformin for GDM: follow up data from the MiG trial. *BMC Pediatr*,15: 54.

Bauman, W.A., Shaw, S., Jayatilleke, E., Spungen, A.M., and Herbert, V. (2000) Increased intake of calcium reverses vitamin B12 malabsorption induced by metformin. *Diabetes Care,* 23(9): 1227-1231.

Bawazeer, N.M. (2011) Vitamin B12 and folate status during pregnancy among Saudi population. UK, Warwick Medical School.

Bell, R., Hayes, L., Lewis-Barned, N., Bilous, M., Brandon, H., Pearson, P., Emmerson, C., Adair, S., Crowder, D., and Bilous, R. (2010) Diagnosis, treatment and outcome of gestational diabetes: A multi-centre study in north-east England (NorGES). *Diabetic Medicine,*27(2 SUPPL. 1).

Bender, D.A. (2003). Folate and other pterins and vitamin B12. *Nutritional Biochemistry of the vitamins*. Cambridge, UK., Cambridge Univ Press: 270-323.

Bertini, A.M., Silva, J.C., Narciso, D.R.R. and Coral, M.L. (2011) Comparative study between metformin and glibenclamide in the treatment of gestational diabetes mellitus. *Diabetes Technology and Therapeutics. Conference: 4th International Conference on Advanced Technologies and Treatments for Diabetes, ATTD,*13(2).

Bertini, A.M., Silva, J.C., Narciso, D.R.R., and Coral, M.L. (2011) Metformin and gestational diabetes mellitus: Maternal factors related to successful treatment. *Diabetes Technology and Therapeutics. Conference: 4th International Conference on Advanced Technologies and Treatments for Diabetes, ATTD,*13(2): 206-207.

Bhate, V., Deshpande, S., Bhat, D., Joshi, N., Ladkat, R., Watve, S., Fall, C., de Jager, C.A., Refsum, H. and Yajnik, C. (2008) Vitamin B12 status of pregnant Indian women and cognitive function in their 9-year-old children. *Food & Nutrition Bulletin,* 29(4): 249-254.

BNF staff. (March, 2015). *British national formulatory*. 69th edition. London, BMJ Group.

Boney, C. M., Verma, A., Tucker, R. and Vohr, B.R. (2005) Metabolic syndrome in childhood: association with birth weight, maternal obesity, and gestational diabetes mellitus. *Pediatrics,* 115(3): e290-296.

Brown, F. M., Wyckoff, J., Rowan, J.A., Jovanovic, L., Sacks, D.A. and Briggs, G.G. (2006) Metformin in pregnancy: its time has not yet come. *Diabetes Care,*29(2): 485-486.

Brown, J.B., Conner, C. and Nicholas, G.A. (2010) Secondary failure of metformin monotherapy in clinical practice. *Diabetes Care,* 33(3): 501-506.

Campbell, I. W. and Ritz, P. (2007) Insights into the effects of metformin on the lipid profile.In *Metformin: The Gold Standard A Scientific Handbook.* Bailey, C.J., Campbell, I.W., Chan, J.C.N., *et al*. UK, Wiley.

Campbell, I.W. and Ritz, P. (2007). Understanding the glucose-lowering actions of metformin. In *Metformin: The Gold Standard A Scientific Handbook.* Bailey, C.J., Campbell, I.W., Chan, J.C.N., *et al*. UK, Wiley.

Canadian Diabetes Association Clinical Practice Guidelines (CDACPG) Expert Committee. (2003) Clinical Practice Guidelines for the Prevention and Management of Diabetes in Canada. *Can J Diabetes,*27:S99-S105

Crowther, C.A., John, J.E.H., Moss, R., McPhee, A.J., Jeffries, W.S., *et al*. (2006). Effect of Treatment of Gestational Diabetes Mellitus on Pregnancy Outcomes. *NEJM*, 352; 2477-2486.

Carpentier, J.L., Bury, J., Luyckx, A. and Lefebvre, P. (1975) Vitamin B12 and Folic acid serum levels in diabetics under various therapeutic regimens. *Diabetes & Metabolism*, 2: 187-190.

Chambers, J. C., Obeid, O.A., Refsum, H., Ueland, P., Hackett, D., Hooper, J., Turner, R.M., Thompson, S.G. and Kooner, J.S. (2000) Plasma homocysteine concentrations and risk of coronary heart disease in UK Indian Asian and European men. *Lancet,*355(9203): 523-527.

Charles, B., Norris, R., Xiao, X.N., and Hague, W. (2006) Population pharmacokinetics of metformin in late pregnancy. *Therapeutic Drug Monitoring*,28(1): 67-72.

Christian, P., Khatry, S.K., Katz, J., Pradhan, E.K., LeClerq, S.C., Shrestha, S.R., Adhikari, R.K., Sommer, A. and West, K.P. (2003) Effects of alternative maternal micronutrient supplements on low birth weight in rural Nepal: double blind randomised community trial. *BMJ*, 326(7389): 571.

Chu, S.Y., Callaghan, W, M., Kim, S.Y., Schmid, C.H., Lau, J., England, L.J. and Dietz, P.M. (2007) Maternal obesity and risk of gestational diabetes mellitus. *Diabetes Care*, 30(8): 2070-2076.

Clausen, T. D., Mathiesen, E.R., Hansen, T., Pedersen, O., Jensen, D.M., Lauenborg, J. and Damm, P. (2008) High prevalence of type 2 diabetes and pre-diabetes in adult offspring of women with gestational diabetes mellitus or type 1 diabetes. *Diabetes Care*, 31(2): 340-346.

Clausson, B., Gardosi, J., Francis, A., and Cnattingius, S. (2001) Perinatal outcome in SGA births defined by customised versus population-based birthweight standards. *BJOG*, 108(8): 830-834.

Coetzee, E.J. and Jackson, W.P.U. (1979) Metformin in Management of Pregnant Insulin-Independent Diabetics. *Diabetologia*, 16: 241-245.

Corbould, A., Swinton, F., Radford, A., Campbell, J., McBeath, S. and Dennis, A. (2013). Fasting blood glucose predicts response to extended-release metformin in gestational diabetes mellitus. *Australian & New Zealand Journal of Obstetrics & Gynaecology*, 53(2): 125-129.

Cundy, T., Ackermann, E. and Ryan, E.A. (2014) Gestational diabetes: new criteria may triple the prevalence but effect on outcomes is unclear. *BMJ*, 348: g1567.

Cutchie, W. A., Cheung, N.W. and Simmons, D. (2006) Comparison of international and New Zealand guidelines for the care of pregnant women with diabetes. *Diabetic Medicine*, 23(5): 460-468.

Dabelea, D. (2010) The Diabetic Intrauterine Environment: Short and Long-term Consequences. In *Gestational Diabetes During and After Pregnancy*. Kim, C. and Ferrara, A. Eds. London, Springer: 227-239.

Damm, P., Kuhl, C., Buschard, K., Jakobsen, B.K., Svejgaard, A., Sodoyez-Goffaux, F.,. Shattock, M., Bottazzo, G.F., and Molsted-Pedersen, L. (1994) Prevalence and predictive value of islet cell antibodies and insulin autoantibodies in women with gestational diabetes. *Diabetic Medicine,* 11(6): 558-563.

de Jager, J., Kooy, A., Lehert, P., Wulffele, M.G., van der Kolk, J., Bets, D., Verburg, J., Donker, J.A., and Stehouwer, C.D. (2010) Long term treatment with metformin in patients with type 2 diabetes and risk of vitamin B-12 deficiency: randomised placebo controlled trial. *BMJ* 340: c2181.

Depeint, F., Bruce, W.R., Shangari, N., Mehta, R. and O'Brien, P.J. (2006) Mitochondrial function and toxicity: role of B vitamins on the one-carbon transfer pathways. *Chem Biol Interact*, 163(1-2): 113-132.

Dornhorst, A., Paterson, C.M., Nicholls, J.S.D., Wadsworth, J., Chiu, D.C., Elkeles, R.S., Johnston, D.G. and Beard, R.W. (1992) High Prevalence of Gestational Diabetes in Women from Ethnic Minority Groups. *Diabetic Medicine*, 9: 820-825.

Duran, A., Saenz, S., Torrejon, M.J., Bordiu, E., Del Valle, L., Galindo, M., Perez, N., Herraiz, M.A., Izquierdo, N., Rubio, M.A., Runkle, I., Perez-Ferre, N., Cusihuallpa, I., Jimenez, S., Garcia de la Torre, N., Fernandez, M.D., Montanez, C., Familiar, C., and Calle-Pascual, A.L. (2014) Introduction of IADPSG criteria for the screening and diagnosis of gestational diabetes mellitus results in improved pregnancy outcomes at a lower cost in a large cohort of pregnant women: the St. Carlos Gestational Diabetes Study. *Diabetes Care*, 37(9): 2442-2450.

Durnwald, C. P., Mele, L., Spong, C.Y., Ramin, S.M., Varner, M.W., Rouse, D.J., Sciscione, A., Catalano, P., Saade, G., Sorokin, Y., Tolosa, J.E., Casey, B., Anderson,

G.D., Eunice, H. National Institute of Child and N. Human Development Maternal-Foetal Medicine Units. (2011) Glycemic characteristics and neonatal outcomes of women treated for mild gestational diabetes. *Obstet Gynecol*, 117(4): 819-827.

Dwarkanath, P., Barzilay, P.R., Thomas, T., Thomas, A., Bhat, S. and Kurpad, A.V. (2013). High folate and low vitamin B-12 intakes during pregnancy are associated with small-for-gestational age infants in South Indian women: a prospective observational cohort study. *Am J Clin Nutr*, 98(6): 1450-1458.

Farrar, D., Fairley, L., Santorelli, G., Tuffnell, D., Sheldon, T.A., Wright, J., Overveld L. and Lawlor, D.A. (2015). Association between hyperglycaemia and adverse perinatal outcomes in south Asian and white British women: analysis of data from the Born in Bradford cohort. The Lancet Diabetes & Endocrinology, 3(10): 795-804.

Feig, D. S. (2008) Metformin therapy for gestational diabetes mellitus: are we there yet? *Nature Clinical Practice Endocrinology & Metabolism*, 4(12): 654-655.

Ferrara, A. (2007). Increasing prevalence of Gestational Diabetes Mellitus. *Diabetes Care*, 30 (Suppl 2): S141-S146.

Foufelle, F. and Ferre, P. (2002) New perspectives in the regulation of hepatic glycolytic and lipogenic genes by insulin and glucose: a role for the transcription factor sterol regulatory element binding protein-1c. *Biochem J*, 366(Pt 2): 377-391.

Fraser, A., Tilling, K., Macdonald-Wallis, C., Sattar, N., Brion, M.J., Benfield, L., Ness, A., Deanfield, J., Hingorani, A., Nelson, S.M., Smith, G.D. and Lawlor, D.A. (2010) Association of maternal weight gain in pregnancy with offspring obesity and metabolic and vascular traits in childhood. *Circulation*; 121(23): 2557-2564.

Gadgil, M., Joshi, K., Pandit, A., Otiv, S., Joshi, R., Brenna, J.T. and Patwardhan, B. (2014) Imbalance of folic acid and vitamin B12 is associated with birth outcome: an Indian pregnant women study. *Eur J Clin Nutr,* 68(6): 726-729.

Galtier, F. (2010) Definition, epidemiology, risk factors. *Diabetes & Metabolism,*36(6 Pt 2): 628-651.

Gandhi, P., Bustani, R., Madhuvrata, P. and Farrell, T. (2012) Introduction of metformin for gestational diabetes mellitus in clinical practice: Has it had an impact? *European Journal of Obstetrics, Gynecology, & Reproductive Biology*,160(2): 147-150.

Gardosi, J. (2004) Customized foetal growth standards: rationale and clinical application. *Semin Perinatol,* 28(1): 33-40.

George, A., Mathews, J.E., Sam, D., Beck, M., Benjamin, S.J., Abraham, A., Antonisamy, B., Jana, A.K., and Thomas, N. (2015) 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*, 55(1): 47-52.

Gilbert, C., Valois, M. and Koren, G. (2006) Pregnancy outcome after first-trimester exposure to metformin: a meta-analysis. *Fertility and Sterility*, 86(3): 658-663.

Goh, J. E. L., Sadler, L. and Rowan, J. (2011) Metformin for gestational diabetes in routine clinical practice. *Diabetic Medicine*, 28(9): 1082-1087.

Gopalakrishnan, V., Singh, R., Pradeep, Y., Kapoor, D., Rani, A.K., Pradhan, S., Bhatia, E. and Yadav, S.B. (2015) Evaluation of the prevalence of gestational diabetes mellitus in North Indians using the International Association of Diabetes and Pregnancy Study groups (IADPSG) criteria. *J Postgrad Med*, 61(3): 155-158.

Hyperglycaemia and adverse pregnancy outcome (HAPO) group. (2008). Hyperglycemia and adverse pregnancy outcomes. *New England Journal of Medicine*, 358(19): 1991-2002.

Guerra-Shinohara, E. M., Paiva, A.A., Rondo, P.H.C., Yamasaki, K., Terzi, C.A. and D'Almeida, V. (2002) Relationship between total homocysteine and folate levels in pregnant women and their newborn babies according to maternal serum levels of vitamin B12. *BJOG: An International Journal of Obstetrics & Gynaecology*, 109(7): 784-791.

Gui, J., Liu, Q. and Feng, L. (2013) Metformin vs insulin in the management of gestational diabetes: a meta-analysis. *PLoS One*, 8(5): e64585.

Gulati, S., Brody, L.C. and Banerjee, R. (1999) Posttranscriptional regulation of mammalian methionine synthase by B12. *Biochem Biophys Res Commun*, 259(2): 436-442.

Gururaj, S., Manuel, C., Canon, T., Belgaumkar, P. and Joseph, S. (2010) Compared to dietary management alone, metformin use in gestational diabetes is associated with lower post-prandial and fasting blood glucose values in the post-natal period. *Diabetic Medicine*, 27(2 SUPPL. 1).

Guven, M. A., Kilinc, M., Batukan, C., Ekerbicer, H.C. and Aksu, K. (2006) Elevated second trimester serum homocysteine levels in women with gestational diabetes mellitus. *Archives of Gynecology & Obstetrics*, 274(6): 333-337.

Hadar, E. and Yogev, Y. (2013) Translating the HAPO study into new diagnostic criteria for GDM? From HAPO to IADPSG and back to O'Sullivan. *Clin Obstet Gynecol*, 56(4): 758-773.

Hague, W. M. (2003). Homocysteine and pregnancy. *Best Practice & Research in Clinical Obstetrics & Gynaecology*, 17(3): 459-469.

Hashmi, F., Malik, A., Sheikh, L. and Ismail, H. (2012) Effectiveness of metformin versus insulin for treating diabetes in pregnancy - A retrospective cohort study to

compare maternal and perinatal outcomes. *BJOG: An International Journal of Obstetrics and Gynaecology*, 119: 95.

Hassan, J. A., Karim, N and Sheikh, Z. (2012). Metformin prevents macrosomia and neonatal morbidity in gestational diabetes. *Pakistan Journal of Medical Sciences*, 28(3): 384-389.

Hayden, H., Taylor, L., Peters, J. and Charlton, M. (2010). Pregnancy outcomes in women with gestational diabetes treated with metformin. *Diabetic Medicine*, 27(2 SUPPL. 1).

Hellmuth, E., Damm, P and Molsted-Pedersen, L. (2000) Oral hypoglycaemic agents in 118 diabetic pregnancies. *Diabetic Medicine*, 17(7): 507-511.

Hermann, L. S., Kalen, J., Katzman, P., Lager, I., Nilsson, A., Norrhamn, O., Sartor, G. and Ugander, L. (2001) Long-term glycemic improvement after addition of metformin to insulin in insulin-treated obese type 2 diabetes patients. *Diabetes, Obesity and Metabolism*, 3: 428-434.

Higgins, J. and Green, S. Eds. (2011). Assessing risk of bias in included studies. The Cochrane Collaboration, UK.

Hogeveen, M., Blom, H.J. and den Heijer, M. (2012) Maternal homocysteine and small-for-gestational-age offspring: systematic review and meta-analysis. *Am J Clin Nutr*, 95(1): 130-136.

Horton, J. D., Goldstein, J.L. and Brown, M.S. (2002). SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J Clin Invest*, 109(9): 1125-1131.

Howlett, H. C. and Bailey, C.J. (1999) A risk-benefit assessment of metformin in type 2 diabetes mellitus. *Drug Safety*, 20(6): 489-503.

Humphrey, L. L., Fu, R., Rogers, K., Freeman, M., and Helfand, M. (2008) Homocysteine level and coronary heart disease incidence: a systematic review and meta-analysis. *Mayo Clin Proc*, 83(11): 1203-1212.

Hunsberger, M., Rosenberg, K.D. and Donatelle, R.J. (2010) Racial/Ethnic Disparities in Gestational Diabetes Mellitus: Findings from population-based survey. *Women's Health Issues*; 20(5): 323-328.

Icayan, E. E. and Chow, B.F. (1962) Effect of vitamin B12 deficiency on cholesterol metabolism. *J Nutr*, 78: 109-114.

IDF Clinical Guidelines Task Force. (2009) *Global Guideline on Pregnancy and Diabetes*. Brussels: International Diabetes Federation.

Iftakhar, R. A., Cheer, K., Wylie, S., Howell, S.J. and Kaushal, K. (2012) Benefit of metformin in reducing weight gain and insulin requirement in pregnancies complicated by gestational diabetes. *Diabetic Medicine*, 29: 26.

Ijas, H., Vaarasmaki, M., Morin-Papunen, L., Keravuo, R., Ebeling, T., Saarela, T., and Raudaskoski, T. (2011) Metformin should be considered in the treatment of gestational diabetes: a prospective randomised study. *BJOG-an International Journal of Obstetrics and Gynaecology*, 118(7): 880-885.

Ijas, H., Vaarasmaki, M., Saarela, T., Keravuo, R., and Raudaskoski, T. (2015) A follow-up of a randomised study of metformin and insulin in gestational diabetes mellitus: growth and development of the children at the age of 18 months. *BJOG*, 122(7): 994-1000.

International Diabetes Federation. (2014) IDF Diabetes Atlas, 6th edn revision. Brussels, Belgium, International Diabetes Federation.

Jawa, A. A., Akram, J., Sultan, M., Humayoun, M.A. and Raza, R. (2010) Nutrition-related vitamin b12 deficiency in patients in pakistan with type 2 diabetes mellitus not taking metformin. *Endocrine practice*, 16(2): 205-208.

Jovanovic, L. (2007). Point: Oral hypoglycemic agents should not be used to treat diabetic pregnant women. *Diabetes Care*, 30(11): 2976-2979.

Jovanovic, L. and D. J. Pettitt (2007). Treatment with insulin and its analogs in pregnancies complicated by diabetes. *Diabetes Care*, 30(Suppl 2): S220-224.

Kahramaner, Z., Sutcuoglu, S., Cicek, E., Erdemir, A., Cosar, H., Turkoglu, E., Colak, A. and Ozer, E.A. (2013) Homocysteine levels and its association with intraventricular hemorrhage in preterm infants born to preeclamptic mothers. *J Matern Foetal Neonatal Med*, 26(18): 1833-1837.

Kalhan, S. C. (2009). Metabolism of methionine in vivo: impact of pregnancy, protein restriction, and fatty liver disease. *Nestle Nutrition Workshop Series. Paediatric Programme,*63: 121-131; discussion 131-123, 259-168.

Kamely, D., Littlefield, J.W. and Erbe, R.W. (1973) Regulation of 5-methyltetrahydrofolate: homocysteine methyltransferase activity by methionine, vitamin B12, and folate in cultured baby hamster kidney cells. *Proc Natl Acad Sci U S A*, 70(9): 2585-2589.

Kersten, S. (2001) Mechanisms of nutritional and hormonal regulation of lipogenesis. *EMBO Rep*, 2(4): 282-286.

Khaire, A., Rathod, R., Kale, A. and Joshi, S. (2015) Vitamin B12 and omega-3 fatty acids together regulate lipid metabolism in Wistar rats. *Prostaglandins Leukot Essent Fatty Acids*, 99: 7-17.

Khandanpour, N., Loke, Y.K., Meyer, F.J., Jennings, B. and Armon, M.P. (2009) Homocysteine and peripheral arterial disease: systematic review and meta-analysis. *Eur J Vasc Endovasc Surg*, 38(3): 316-322.

Kiel, D. W.,Dodson, E.A., Artal, R., Boehmer, T.K. and Leet, T.L. (2007) Gestational Weight Gain and Pregnancy Outcomes in Obese Women: How much Is Enough? *Obstetrics & Gynaecology*, 110(4): 752-758.

Kilicdag, E. B., Bagis, T., Tarim, E., Aslan, E., Erkanli, S., Simsek, E., Haydardedeoglu, B. and Kuscu E. (2005) Administration of B-group vitamins reduces circulating homocysteine in polycystic ovary syndrome patients treated with metformin: a randomized trial. *Human Reproduction*, 20(6): 1521-1528.

Kim, M. and J. R. Wands (2010) Insulin Pathway. *Signaling Pathways in Liver Disease.* New York, NY, Springer-Verlag.

Kingsbury, K. J. and Bondy, G. (2003) Understanding the essentials of blood lipid metabolism. *Prog Cardiovasc Nurs*, 18(1): 13-18.

Kirke, P. N., Molloy, A.M.,Daly, L.E.,Burke, H.,Weir, D.C.,Scott, J.M. (1993). Maternal plasma folate and vitamin B12 are independent risk factors for neural tube defects. *QJM* 86(11): 703-708.

Kitwitee, P., Limwattananon, S., Limwattananon, C., Waleekachonlert, O., Ratanachotpanich, T., Phimphilai, M., Nguyen, T.V. and Pongchaiyakul, C. (2015) Metformin for the treatment of gestational diabetes: An updated meta-analysis. *Diabetes Res Clin Pract*, 62:1522-1534.

Kitzmiller, J. L. (2010). An overview of Problems and Solutions in the Diagnosis and Treatment of Gestational Diabetes. *Gestational Diabetes During and After Pregnancy*. Kim, C and Ferrara, A, Eds. London, Springer: 1-9.

Knopp, R. H., Chan, E., Zhu, X., Paramsothy, P. and Bonet, B. Eds. (2010) Lipids in Gestational Diabetes: Abnormalities and Significance. *Gestational Diabetes During and After Pregnancy*. London, Springer.

Knudtson, E. J., Smith, K., Mercer, B.M., Miodovnik, M., Thurnau, G.R., Goldenberg, R.L., Meis, P.J., Moawad, A.H., Vandorsten, J.P., Sorokin, Y., Roberts, J.M., Das, A.., *et al*. National Institutes of Child and N. Human Development Maternal-Foetal Medicine Units (2004) Serum homocysteine levels after preterm premature rupture of the membranes. *Am J Obstet Gynecol*, 191(2): 537-541.

Koplay, M., Gulcan, E and Ozkan, F. (2011) Association between serum vitamin B12 levels and the degree of steatosis in patients with nonalcoholic fatty liver disease. *J Investig Med*, 59(7): 1137-1140.

Krishnaveni, G. V., Hill, J.C., Veena, S.R., Bhat, D.S., Wills, A.K., Karat, C.L., Yajnik, C.S. and Fall, C.H. (2009) Low plasma vitamin B12 in pregnancy is associated with gestational 'diabesity' and later diabetes. *Diabetologia*,52(11): 2350-2358.

Krishnaveni, G. V., Veena, S.R., Karat, S.R., Yajnik, C.S., and Fall, C.H.D. (2014) Association between maternal folate concentrations during pregnancy and insulin resistance in Indian children. *Diabetologia*, 57(1): 110-121.

Kumar, K. A., Lalitha, A., Pavithra, D., Padmavathi, I.J., Ganeshan, M., Rao, K.R., Venu, L., Balakrishna, N., Shanker, N.H., Reddy, S.U., Chandak, G.R., Sengupta, S. and Raghunath, M. (2013) Maternal dietary folate and/or vitamin B12 restrictions alter body composition (adiposity) and lipid metabolism in Wistar rat offspring. *J Nutr Biochem*, 24(1): 25-31.

Kwan, L. L., Bermudez, O.I. and Tucker, K.L. (2002). Low vitamin B-12 intake and status are more prevalent in Hispanic older adults of Caribbean origin than in neighborhood-matched non-Hispanic whites. *Journal of Nutrition*, 132(7): 2059-2064.

Lai, J., Tan, J., Cheng, Y., *et al*. (2008) Cost analysis of metformin vs insulin in management of gestational diabetes. *American Journal of Obstetrics & Gynecology*: S231.

Landon, M. B., Spong, C.Y., Thom, E., Carpenter, M.W., Ramin, S.M., Casey, B., Wapner, R.J., Varner, M.W., Rouse, D.J., Thorp, J.M. Sciscione, J.A., Catalano, P.,

Harper, M., Saade, G., Lain, K.Y., Sorokin, Y., Peaceman, A.M., Tolosa, J.E., Anderson, J.B., Eunice, H. National Institute of Child and N. Human Development Maternal-Foetal Medicine Units (2009). A multicenter, randomized trial of treatment for mild gestational diabetes. *N Engl J Med,* 361(14): 1339-1348.

Langer, O. (2008). Gestational Diabetes: The consequences of not-treating. *Textbook of Diabetes and Pregnancy*. M. Hod, Jovanovic, L., Renzo, G.C.D., Leiva, A.D. and Langer, O. London, Informa Healthcare: 107-117.

Lapolla, A., Dalfra, M.G., Ragazzi, E., De Cata, A.P., and Fedele, D. (2011) New International Association of the Diabetes and Pregnancy Study Groups (IADPSG) recommendations for diagnosing gestational diabetes compared with former criteria: a retrospective study on pregnancy outcome. *Diabetic Medicine, 2*8(9): 1074-1077.

Lautatzis, M. E., Goulis, D.G. and Vrontakis, M. (2013) Efficacy and safety of metformin during pregnancy in women with gestational diabetes mellitus or polycystic ovary syndrome: a systematic review. *Metabolism*, 62(11): 1522-1534.

Lawrence, J. M. (2010). Prevalence of GDM. *Gestational Diabetes During & After Pregnancy*. Kim, C and Ferrara, A. London., Springer.: 53-69.

Lee, J. O., Lee, S.K., Jung, J.H., Kim, J.H., You, G.Y., Kim, S.K., Park, S.H., Uhm, K.O. and Kim, H.S. (2011) Metformin induces Rab4 through AMPK and modulates

GLUT4 translocation in skeletal muscle cells. *Journal of Cellular Physiology,* 226(4): 974-981.

Lee, S., Lee,M.S., Kim, C.T., Kim, I.H., and Kim, Y. (2012) Ginsenoside Rg3 Reduces Lipid Accumulation with AMP-Activated Protein Kinase (AMPK) Activation in HepG2 Cells. *Int J Mol Sci,* 13(5): 5729-5739.

Li, Y., Xu, S., Mihaylova, M.M., Zheng, B., Hou, X., Jiang, B., Park, O., Luo, Z., Lefai, E., Shyy, J.T., Gao, B., Wierzbicki, M., Verbeuren, T.J., Shaw, R.J., Cohen, R.A., and Zang, M (2011). AMPK phosphorylates and inhibits SREBP activity to attenuate hepatic steatosis and atherosclerosis in diet-induced insulin-resistant mice. *Cell Metab,*13(4): 376-388.

Liao, S., Mei, J., Song, W., Liu, Y., Tan, Y.D., Chi, S., Li, P., Chen, X. and Deng, S. (2014) The impact of the International Association of Diabetes and Pregnancy Study Groups (IADPSG) fasting glucose diagnostic criterion on the prevalence and outcomes of gestational diabetes mellitus in Han Chinese women. *Diabet Med*, 31(3): 341-351.

Liberati, A., Altman, D., Tetzlaff, J., Mulrow, C., Gotzsche, P., Ioannidis, J., Clarke, M., Devereaux, P., Kleijnen, J. and Moher, D. (2009) The PRISMA Statement for Reporting Systematic Reviews and Meta-analyses of Studies that evaluate health care interventions: Explanation and Elaboration. *Annuals of Internal Medicine*, 151(4): W 65 - W93.

Lilly, M. and Godwin, M. (2009) Treating prediabetes with metformin systematic review and meta-analysis. *Canadian Family Physician,* 55(4): 363-369.

Lindsay, J. R., Duffy, N.A., McKillop, A.M., Ardill, J., O'Harte, F.P., Flatt, P.R., and Bell, P.M. (2005) Inhibition of dipeptidyl peptidase IV activity by oral metformin in Type 2 diabetes. *Diabetic Medicine,*22(5): 654-657.

Lindsay, J. R., Duffy, N.A., McKillop, A.M., Ardill, J., O'Harte, F.P.M., Flatt, P.R., and Bell, P.M. (2005) Inhibition of dipeptidyl peptidase IV activity by oral metformin in Type 2 diabetes. *Diabetes Medicine,* 22: 654-657

Liu, K. W., Dai, L.K. and Jean, W. (2006) Metformin-related vitamin B12 deficiency. *Age and Ageing,* 35(2): 200-201.

Lord, J., Thomas, R., Fox, B., Acharya, U and Wilkin, T. (2006) The effect of metformin on fat distribution and the metabolic syndrome in women with polycystic ovary syndrome--a randomised, double-blind, placebo-controlled trial. *BJOG: An International Journal of Obstetrics & Gynaecology,*113(7): 817-824.

Lord, J. M., Flight, I.H. and Norman, R.J. (2003) Metformin in polycystic ovary syndrome: systematic review and meta-analysis. *BMJ (Clinical research ed.),* 327(7421): 951-953.

MacFarlane, A. J., Greene-Finestone, L.S. and Shi, Y. (2011) Vitamin B-12 and homocysteine status in folate-replete population: results from the Canadian Health Measures Survey. *American Journal of Clinical Nutrition*, 94(4): 1079-1087.

Mahalle, N., Kulkarni, M.V., Garg, M.K. and Naik, S.S. (2013). Vitamin B12 deficiency and hyperhomocysteinemia as correlates of cardiovascular risk factors in Indian subjects with coronary artery disease. *J Cardiol*, 61(4): 289-294.

Mangoni, A. A., Sherwood, R.A., Asonganyi, B., Ouldred, E.L., Thomas, S. and Jackson, S.H. (2005). Folic acid: a marker of endothelial function in type 2 diabetes?. *Vasc Health Risk Manag*,1(1): 79-83.

Landon, M.B., Elizabeth, C.Y.S., Marshall, W., Susan, W., Ramin, M *et al*. (2009). A Multicenter, Randomized Trial of Treatment for Mild Gestational Diabetes. *The New England Journal of Medicine*, 361(14): 1339-1348.

Piccole, M., Abdulhaj Martinez, S.M., Andres Nunez, P., Garcia Leon, P., Lopez Sanchez, E.J. and A. R. Gonzalez Ramirez (2010). A randomized study comparing metformin and insulin in the treatment of gestational diabetes mellitus. interim results. *Journal of Maternal-Foetal and Neonatal Medicine*, 23: 381.

McCurdy, C. and Friedman, J. (2010) Mechanisms Underlying Insulin Resistance in Human Pregnancy and Gestational Diabetes Mellitus. *Gestational Diabetes During and After Pregnancy*. Kim, C and Ferrara, A. London, Springer: 125-138.

Meek, C. L., Lewis, H.B., Patient, C., Murphy, H.R., and Simmons, D. (2015). Diagnosis of gestational diabetes mellitus: falling through the net. *Diabetologia*,

Meigs, J. B., Jacques, J.F., Selhub, J., Singer, D.E., Nathan, D.M., Rifai, N., D'Agostino, R.B. and Wilson, P.W.F. (2001) Fasting Plasma Homocysteine levels in the Insulin resistance syndrome. *Diabetes Care*, 24(8): 1403-1410.

Mesdaghinia, E., Samimi, M., Homaei, Z., Saberi, F., Moosavi, S.G. and Yaribakht, M. (2013). Comparison of newborn outcomes in women with gestational diabetes mellitus treated with metformin or insulin: a randomised blinded trial. *Int J Prev Med*, 4(3): 327-333.

Metzger, B. E., Buchanan, T.A., Coustan, D.R., De Leiva, A., Dunger, D.B., Hadden, D.R., Hod, M., Kitzmiller, J.L., Kjos, S.L., Oats, J.N., Pettitt, D.J., Sacks, D.A. and Zoupas, C. (2007) Summary and recommendations of the Fifth International Workshop-Conference on Gestational Diabetes Mellitus. *Diabetes Care*, 30(SUPPL. 2): S251-S260.

Mikolajczyk, R. T., Zhang, J., Betran, A.P., Souza, J.P., Mori, R., Gülmezoglu, A.M., and Merialdi, M. (2011) A global reference for foetal-weight and birthweight percentiles. *Lancet*, 377(9780): 1855-1861.

Miller, R. A., Chu, Q, Xie, J., Foretz, M., Viollet, B. and Birnbaum, M.J. (2013). Biguanides suppress hepatic glucagon signalling by decreasing production of cyclic AMP. *Nature,* 494(7436): 256-260.

Moore, J. H. and Doran, B.M. (1962) Lipid metabolism in the normal and vitamin B12-deficient chick embryo. *Biochem J,* 84: 506-513.

Moore, L. E., Briery, C.M., Clokey, D., Martin, R.M., Williford, N.J., Bofill, J.A. and Morrison, J.C. (2007). Metformin and insulin in the management of gestational diabetes mellitus - Preliminary results of a comparison. *Journal of Reproductive Medicine,* 52(11): 1011-1015.

Moore, L. E., Clokey, D., Rappaport, V.J. and Curet, L.B. (2010). Metformin Compared With Glyburide in Gestational Diabetes A Randomized Controlled Trial. *Obstetrics and Gynecology*, 115(1): 55-59.

Moretti, M. E., Rezvani, M and Koren, G. (2008) Safety of glyburide for gestational diabetes: A meta-analysis of pregnancy outcomes. *Annals of Pharmacotherapy*, 42(4): 483-490.

National Collaborating Centre for Women's and Children's Health. (2015) *NICE clinical 63: Diabetes in pregnancy: Management of diabetes and its complications from pre-conception to the post-natal period.* London: National Institute for Health and Clinical Excellence.

Nguyen, P., Leray, V., Diez, M., Serisier, S., Le Bloc'h, J., Siliart, B. and Dumon, H. (2008) Liver lipid metabolism. *J Anim Physiol Anim Nutr (Berl)*; 92(3): 272-283.

Nielsen, M. J., Rasmussen, M.R., Andersen, C.B., Nexo, E., and Moestrup, S.K. (2012). Vitamin B12 transport from food to the body's cells--a sophisticated, multistep pathway. *Nat Rev Gastroenterol Hepatol*, 9(6): 345-354.

Nightingale, C. M., Rudnicka, A.R., Owen, C.G., Cook, D.G., and Whincup, P.H. (2011) Patterns of body size and adiposity among UK children of South Asian, black African-Caribbean and white European origin: Child Heart And health Study in England (CHASE Study). *International Journal of Epidemiology*, 40(1): 33-44.

Niromanesh, S., Alavi, A., Sharbaf, F.R., Amjadi, N., Moosavi, S. and Akbari, S. (2012) Metformin compared with insulin in the management of gestational diabetes mellitus: A randomized clinical trial. *Diabetes Research and Clinical Practice*, 98(3): 422-429.

Oken, E., Taveras, E.M., Kleinman, K.P., Rich-Edwards, J.W. and Gillman, M.W. (2007) Gestational weight gain and child adiposity at age 3 years. *American Journal of Obstetrics & Gynecology*; 196(4): 322.e321-328.

Oltean, S. and Banerjee, R (2003). Nutritional modulation of gene expression and homocysteine utilization by vitamin B12. *J Biol Chem*, 278(23): 20778-20784.

Omu, A. (2014). Pro-Inflammatory Cytokines, Lipid Metabolism and Inflammation in Gestational Diabetes Mellitus as Cause of Insulin Resistance. *Gestational Diabetes - Causes, Diagnosis and Treatment;* 79-102.

Owen, M. R., Doran, E. and Halestrap, A.P. (2000). Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. *Biochem. J.* 348: 607-614.

Pernicova, I. and Korbonits, M. (2014) Metformin--mode of action and clinical implications for diabetes and cancer. *Nat Rev Endocrinol*, 10(3): 143-156.

Pirkola, J., Pouta, A., Bloigu, A., Hartikainen, A-L., Laitinen, J., Jarvelin, M.R., and Vaarasmaki, M. (2010) Risks of overweight and abdominal obesity at age 16 years asssociated with prenatal exposures to maternal prepregnancy overweight and gestational diabetes. *Diabetes Care*, 33(5): 1115-1121.

Poirier, S., Samami, S., Mamarbachi, M., Demers, A., Chang, T.Y., Vance, D.E., Hatch, G.M. and Mayer, G. (2014) The Epigenetic Drug 5-Azacytidine Interferes with Cholesterol and Lipid Metabolism. *Journal of Biological Chemistry*, 289: 18736-18751.

Pongchaidecha, M., Srikusalanukul, V., Chattananon, A. and Tanjariyaporn, S. (2004) Effect of metformin on plasma homocysteine, vitamin B12 and folic acid: a cross-

sectional study in patients with type 2 diabetes mellitus. *Journal of The Medical Association of Thailand*, 87(7): 780-787.

Postic, C. and Girard, J. (2008). Contribution of de novo fatty acid synthesis to hepatic steatosis and insulin resistance: lessons from genetically engineered mice. *J Clin Invest*, 118(3): 829-838.

Raddatz, D. and Ramadori, G. (2011) Hepatic Carbohydrate Metabolism. *Molecular Pathology of Liver Disease*. London, UK., Springer Science and Business Media: 109-123.

Radfar, M., Jabei, M.D., Hadjibabaie, M. and Barijani, B. (2011). Vitamin B12 levels in type 2 diabetic patients receiving metformin. *International Journal of Clinical Pharmacy*. 33(2): 339.

Rai, L., Meenakshi, D. and Kamath, A. (2009). Metformin--a convenient alternative to insulin for Indian women with diabetes in pregnancy. *Indian Journal of Medical Sciences*, 63(11): 491-497.

Ralph, G. and Joshua, M.W.(2005) Vitamin B12 deficiency is the dominant nutritional cause of hyperhomocysteinemia in a folic acid-fortified population. *Clinical Chemical Laboratory Medicine*, 43(10): 1048-1051.

Reinstatler, L., Qi, Y.P., Williamson, R.S., Garn, J.V. and Oakley, J.P. Jr. (2012) Association of biochemical B(1)(2) deficiency with metformin therapy and vitamin B(1)(2) supplements: the National Health and Nutrition Examination Survey, 1999-2006. *Diabetes Care,* 35(2): 327-333.

Relton, C. L., Pearce, M.S. and Parker, L. (2005) The influence of erythrocyte folate and serum vitamin B12 status on birth weight. *Br J Nutr*, 93(5): 593-599.

Rena, G., Pearson, E.R. and Sakamoto, K. (2013). Molecular mechanism of action of metformin: old or new insights?. *Diabetologia,* 56(9): 1898-1906.

Retnakaran, R., Hanley, A.J.G., Raif, N., Connelly, P.W., Sermer, P. and Zinman, B. (2004) Hypoadiponectinaemia in South Asian women during pregnancy: evidence of ethnic variation in adiponectin concentration. *Diabetic Medicine,* 21(4): 388-392.

Retnakaran, R., Hanley, A.J.G., Connelly, P.W., Sermer, M., and Zinman, B. (2006) Ethnicity modifies the effect of obesity on insulin resistance in pregnancy: a comparison of Asian, South Asian, and Caucasian women. *Journal of Clinical Endocrinology & Metabolism*, 91(1): 93-97.

Retnakaran, R. (2010). Inflammation, Adipokines, and Gestational Diabetes Mellitus. *Gestational Diabetes During and After Pregnancy*. Kim, C. and Ferrara, A. London, Springer: 139-153.

Rowan, J. A., Rush, E.C., Obolonkin, V., Battin, M., Wouldes, T. and Hague, W.M. (2011) Metformin in gestational diabetes: The offspring follow-up (MiG TOFU) - Body composition at 2 years of age. *Diabetes Care,* 34(10): 2279-2284.

Rowan, J. A., Hague, W.M., Gao, W., Battin, M.R. and Moore, M.P. (2008) Metformin versus insulin for the treatment of gestational diabetes. *New England Journal of Medicine,* 358(19): 2003-2015

Rowan, J. A., Gao, W., Hague, W.M. and McIntyre, H.D. (2010). Glycemia and its relationship to outcomes in the metformin in gestational diabetes trial. *Diabetes Care,* 33(1): 9-16.

Ruderman, N. B. and A. K. Saha (2006). "Metabolic Syndrome: Adenosine Monophosphate-activated Protein Kinase and Malonyl Coenzyme A." Obesity 14: 25S-33S.

Ruholamin, S., Eshaghian, S and Allame, Z. (2014) Neonatal outcomes in women with gestational diabetes mellitus treated with metformin in compare with insulin: A randomized clinical trial. *J Res Med Sci,* 19(10): 970-975.

Sacks, D. A., Hadden, D.R., Maresh, M., Deerochanawong, C., Dyer, A.R., Metzger, B.E., Lowe, L.P., Coustan, D.R., Hod, M., Oats, J.J., Persson, B., Trimble, E.R. and H. S. C. R. Group (2012). Frequency of gestational diabetes mellitus at collaborating

centers based on IADPSG consensus panel-recommended criteria: the Hyperglycemia and Adverse Pregnancy Outcome (HAPO) Study. *Diabetes Care*, 35(3): 526-528.

Sahin, M., Tutuncu, N.P., Ertugrul, D., Tanaci, N. and Guvener, N.D. (2007). Effects of metformin or rosiglitazone on serum concentrations of homocysteine, folate and vitamin B12 in patients with type 2 diabetes mellitus. *Journal of Diabetes and its complications*, 21(2): 118-123.

Sakar, Y., Meddah, B., Faouzi, M.A., Cherrah, Y., Bado, A. and Ducroc, R. (2010). Metformin-induced regulations of intestinal D-glucose transporters. *Journal of Physiology and Pharmacology*, 61(3): 301-307.

Saravanan, P. and Yajnik, C. (2010) Role of maternal vitamin B12 on the metabolic health of the offspring: a contributor to the diabetes epidemic? *The British Journal of Diabetes and Vascular Disease*, 10(3): 109-114.

Sardesai, V. (2011). *Water-Soluble Vitamins II : Vitamin B12*. Boca Raton, Taylor & Francis.

Savitz, D. A., Janevic, T.M., Engel, S.M., Kaufman, J.S. (2008). Ethnicity and gestational diabetes in New York City, 1995-2003. *BJOG*, 115: 969-978.

Scarpello, J. H. and Howlett, H.C. (2008) Metformin therapy and clinical uses. *Diab Vasc Dis Res*, 5(3): 157-167.

Scarpello, J. H. B. (2001). Optimal dosing strategies for maximizing the clinical response to metformin in type 2 diabetes. *The British Journal of Diabetes and Vascular Disease*, 1: 28-36.

Schachter, M., Raziel, A., Friedler, S., Strassburger, D., Bern, O. and Ron-El, R. (2003) . Insulin resistance in patients with polycystic ovary syndrome is associated with elevated plasma homocysteine. *Human Reproduction*, 18(4): 721-727.

Schultes, B., Oltmanns, K.M., Kern, W., Fehm, H.L., Born, J. and Peters, A. (2003) Modulation of hunger by plasma glucose and metformin. *Journal of Clinical Endocrinology and Metabolism*, 88(3): 1133-1141.

Schulz, K. F. and Grimes, D.A. (2005). Epidemiology 5: Multiplicity in randomized trials II: subgroup and interim analyses. *Lancet*, 365: 1657-1661.

Scottish Intercollegiate Guidelines Network: (2010) *Management of diabetes : A national clinical guideline* (116). Edinburgh: NHS Quality improvement Scotland; 56-69.

Seghieri, G., Breschi, M.C., Anichini, R., De Bellis, A., Alviggi, L., Maida, I. and Franconi, F. (2003) Serum homocysteine levels are increased in women with gestational diabetes mellitus. *Metabolism: Clinical & Experimental.* 52(6): 720-723.

Selhub, J., Morris, M.S. and Jacques, P.F. (2007). In vitamin B12 deficiency, higher serum folate is associated with increased total homocysteine and methylmalonic acid concentrations. *Proc Natl Acad Sci U S A,* 104(50): 19995-20000.

Seshlah, V., Sahay, B.K., Das, A.D., Shah, S., Banerjee, S., Rao, P.V., Ammini, A., Balaji, V., Gupta, S., Divakar, H., Misra, S. and Thanawala, U. (2009) Gestational diabetes mellitus - Indian guidelines. *Journal of the Indian Medical Association,* 107(11): 799-806.

Siddaramaiah, N., Taylor, R., Marshall, S,M., Waugh, J., Davison, J.M. and Forsey, J. (2011). Metformin in gestational diabetes: Benefits on maternal weight? *Diabetic Medicine,* 28: 177.

Silva, J. C., Fachin, N., Coral, M.L. and Bertini, A.M. (2012). Perinatal impact of the use of metformin and glyburide for the treatment of gestational diabetes mellitus. *Journal of Perinatal Medicine,* 40(3): 225-228.

Silva, J. C., Pacheco, C., Bizato, J., de Souza, B.V., Ribeiro, T.E., and Bertini, A.M. (2010). Metformin compared with glyburide for the management of gestational diabetes. *International Journal of Gynecology & Obstetrics,* 111(1): 37-40.

Silva, R. M., Fontes, A.C., Silva, K.A., Sant'Ana, T.A., Ramos, F.J., Marques-Salles Tde, J., Pombo-de-Oliveira, M.S. and Muniz, M.T. (2013) Polymorphisms involved

in folate metabolism pathways and the risk of the development of childhood acute leukemia. *Genet Test Mol Biomarkers*. 17(2): 147-152.

Simmons, D., McElduff, A., McIntyre, H.D. and Elrishi, M. (2010). Gestational diabetes mellitus: NICE for the U.S.? A comparison of the American Diabetes Association and the American College of Obstetricians and Gynecologists guidelines with the U.K. National Institute for Health and Clinical Excellence guidelines. *Diabetes Care*, 33(1): 34-37.

Singh, S. K. and Rastogi, A. (2008) Gestational diabetes mellitus. *Diabetes and Metabolic Syndrome: Clinical Research and Reviews*, 2(3): 227-234.

Soper, D. S. (2014). Post-hoc Statistical Power Calculator for Multiple Regression. https://www.danielsoper.com/statcalc.

Sorich, W., Stranks, S.N., Kowalski, S.R., Kuruvila, M.T. and Sorich, M.J. (2008) Vitamin B12 deficiency in the elderly using metformin long term: Prevalence and relationship to putative risk factors. *Journal of Pharmacy Practice and Research*, 38(2): 111-113.

Spaulonci, C. P., Bernardes, L.S., Trindade, C. and Zugaib, M.. (2013). Randomized trial of metformin vs insulin in the management of gestational diabetes. *American Journal of Obstetrics & Gynecology* 208: 1.e1-1.e7.

Stewart, C. P., Christian, P., Schulze, K.J., Arguello, M., LeClerq, S.C., Khatry, S.K., and West, K.P. (2011) Low maternal vitamin B-12 status is associated with offspring insulin resistance regardless of antenatal micronutrient supplementation in rural Nepal, *J Nut.* 141(10): 1912-1917.

Su, D. F. and Wang, X.Y. (2014). Metformin vs insulin in the management of gestational diabetes: a systematic review and meta-analysis. *Diabetes Res Clin Pract*, 104(3): 353-357.

Sudchada, P., Saokaew, S., Sridetch, S., Incampa, S., Jaiyen, S. and Khaithong, W. (2012) Effect of folic acid supplementation on plasma total homocysteine levels and glycemic control in patients with type 2 diabetes: a systematic review and meta-analysis. *Diabetes Res Clin Pract*, 98(1): 151-158.

Sukla, K. K., Tiwari, P.K., Kumar, A. and Raman, R. (2013) Low birthweight (LBW) and neonatal hyperbilirubinemia (NNH) in an Indian cohort: association of homocysteine, its metabolic pathway genes and micronutrients as risk factors. *PLoS One*, 8(8): e71587.

Tam WH, Ma, R., Yang, X., Li, A., Ko, G., Kong, A., Lao, T., Chan, M., Lam, C. and Chan, J (2010). Glucose intolerance and cardiometabolic risk in adolescents exposed to maternal gestational diabetes. *Diabetes Care*, 33(6): 1382-1384.

Tertti, K., Ekblad, U., Vahlberg, T. and Ronnemaa, T. (2008) Comparison of metformin and insulin in the treatment of gestational diabetes: a retrospective, case-control study. *Rev Diabet Stud,* 5(2): 95-101.

Tertti, K., Ekblad, U., Koskinen, P., Vahlberg, T and Ronnemaa, T. (2013) Metformin vs. insulin in gestational diabetes. A randomized study characterizing metformin patients needing additional insulin. *Diabetes Obesity & Metabolism,* 15(3): 246-251.

Tertti, K., Laine, K., Ekblad, U, Rinne, V., Ronnemaa, T. (2014) The degree of foetal metformin exposure does not influence foetal outcome in gestational diabetes. *Acta Diabetol*, 51(5);731-8.

Tessari, P., A. Coracina, A. Cosma and A. Tiengo (2009). "Hepatic lipid metabolism and non-alcoholic fatty liver disease." Nutr Metab Cardiovasc Dis 19(4): 291-302.

Ting, R. Z. W., Szeto, C.C., Chan, M.H.M., Ma, K.K. and Chow, K.M. (2006) Risks factors of vitamin B12 deficiency in patients receiving metformin. *Archives of Internal Medicine,* 166: 1975-1979.

Tomkin, G. H., Hadden, D.R., Weaver, J.A. and Montgomery, D.A.D. (1971) Vitamin-B12 Status of Patients on long-term metformin therapy. *British Medical Journal*, 2: 685-687.

UK Prospective Diabetes Study (UKPDS) Group. (1998) Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes. *Lancet,* 352(9131): 854-865.

Vanky, E., Stridsklev, S., Heimstad, R., Romundstad, P., Skogoy, K., Kleggetveit, O., Von Brandis, P., Eikeland, T., Flo, K., Berg, K.F., Bunford, G., Lund, A., Bjerke, C., Almas, I., Berg, H.A, Danielson, A., Lahmami, G. and Carlsen, S.M. (2010). "Metformin Versus placebo from first trimester to delivery in polycystic ovary syndrome: A randomized, controlled multicenter study." Journal of Clinical Endocrinology and Metabolism 95(12): E448-E455.

Veena, S. R., Krishnaveni, G.V., Srinivasan, K., Kurpad, A.V., Muthayya, S., Hill, J.C., Kiran, K.N. and Fall, C.H.D. (2010). Childhood cognitive ability: relationship to gestational diabetes mellitus in India. *Diabetologia,* 53(10): 2134-2138.

Wong, V. W. and Jalaludin, B. (2011). Gestational diabetes mellitus: who requires insulin therapy? Australian & New Zealand Journal of Obstetrics & Gynaecology, 51(5): 432-436.

Wulffele, M. G., Kooy, A., de Zeeuw, D., Stehouwer, C.D. and Gansevoort, R.T. (2004). The effect of metformin on blood pressure, plasma cholesterol and triglycerides in type 2 diabetes mellitus: a systematic review. Journal of Internal Medicine, 256(1): 1-14.

Wulffele, M. G., Kooy, A., Lehert, L., Bets, D., Ogterop, J.C., Van Borger Der Burg,B., Donker, A.J.M. and Stehouwer, C.D.A. (2003) Effects of short-term treatment with metformin on serum concentrations of homocysteine, folate and vitamin B12 in type 2 diabetes mellitus: A randomized, placebo-controlled trial. *Journal of Internal Medicine*, 254(5): 455-463.

Xie, Z., Dong, Y., Scholz, R., Neumann, D. and Zou, M.H. (2008) Phosphorylation of LKB1 at serine 428 by protein kinase C-zeta is required for metformin-enhanced activation of the AMP-activated protein kinase in endothelial cells. *Circulation*, 117(7): 952-962.

Yajnik, C. S. and Deshmukh, U.S. (2008a). Maternal nutrition, intrauterine programming and consequential risks in the offspring. *Reviews in Endocrine & Metabolic Disorders*, 9(3): 203-211.

Yajnik, C. S., Deshpande, S.S., Jackson, A.A., Refsum, H., Rao, S., Fisher, D.J., Bhat, D.B., Naik, S.S., Coyaji, K.J., Joglekar, C.V., Joshi, N., Lubree, H.G., Deshpande, V.U., Rege, S.S. and Fall, C.H. (2008b). Vitamin B12 and folate concentrations during pregnancy and insulin resistance in the offspring: the Pune Maternal Nutrition Study. *Diabetologia*, 51(1): 29-38.

Yajnik, C. S., Deshpande, S.S., Panchanadikar, A.V., Naik, S.S., Deshpande, J.A., Coyaji, K.J., Fall, C. and Refsum, H. (2005) Maternal total homocysteine concentration and neonatal size in India. Asia Pacific Journal of Clinical Nutrition. 14(2): 179-181.

Yasuda, N., Inoue, T., Nagakura, T., Yamazaki, K., Kira, K., Saeki, T. and Tanaka, I. (2002) Enhanced secretion of glucagon-like peptide 1 by biguanide compounds. *Biochem Biophys Res Commun,* 298(5): 779-784.

Yogev, Y., Ben-Haroush, A. and Hod, M. (2008) Pathogenesis of gestational diabetes mellitus. *Diabetes and Pregnancy.* M. Hod and *et al*. New York, Informa Healthcare: 71-78.

Yue, D. K., Molyneaux, L.M., Ross, G.P., Constantino, M.I., Child, A.G. and Turtle, J.R. (1996). Why does ethnicity affect prevalence of gestational diabetes? The underwater volcano theory. *Diabetic Medicine,* 13(8): 748-752.

Zang, M., Zuccollo, A., Hou, X., Nagata, D., Walsh, K., Herscovitz, H., Brecher, P., Ruderman, N.B., and Cohen, R.A. (2004). AMP-activated protein kinase is required for the lipid-lowering effect of metformin in insulin-resistant human HepG2 cells. *J Biol Chem.* 279(46): 47898-47905.

Zhang, C. (2010). Risk factors for Gestational Diabetes: from an Epidemiological Standpoint. *Gestational Diabetes During and After Pregnancy.* Kim, C and Ferrara, A. London, Springer: 71-81.

Zhou, G., Myers, R., Li, Y., Chen, Y., Shen, X., Fenyk-Melody, J., Wu, M., Ventre, J. and *et al*. (2001). Role of AMP-activated protein kinase in mechanism of metformin action. *The Journal of Clinical investigation*, 108(8): 1167-1174.

Zierz, S. and Engel, A.G. (1987). Different sites of inhibition of carnitine palmitoyltransferase by malonyl-CoA, and by acetyl-CoA, in human skeletal muscle. *Biochem. J*, 245: 205-209.