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ABSTRACT

Gestational diabetes is a common pregnancy complication that increases the risk of adverse pregnancy outcomes and predicts long-term metabolic morbidity for the mother and the offspring. Gestational diabetes is treated with lifestyle modifications and metformin or insulin if needed. Besides hyperglycemia, gestational diabetes is associated with broad disturbances in lipid and amino acid metabolism and low-grade inflammation. The effects of metformin on these changes compared to insulin are not fully known.

In this secondary analysis of a previous randomized trial in gestational diabetes, the effects of metformin (n = 110) and insulin (n = 107) treatments were studied on the maternal metabolome, inflammatory marker profile and insulin-like growth factor-binding protein-1 phosphoisoforms. Patients (n = 126) not requiring antihyperglycemic medication were included as a reference group at the time of randomization to medical treatment groups. Umbilical cord blood samples were drawn after delivery in all three groups to study the effects of metformin on the fetal metabolome.

Metformin treatment led to a greater increase in maternal serum alanine, total triglycerides, very low-density lipoprotein triglycerides and total fatty acids than insulin. In the cord serum metabolome, only alanine was significantly higher in the metformin group. Maternal lipids, very low-density lipoprotein cholesterol and the apolipoprotein B to A-1 ratio in particular, were related to an increased birth weight and these associations were stronger in the metformin group than the insulin group. In cord blood, omega-6 fatty acids were positively and omega-3 fatty acids inversely associated with birth weight. Metformin had no effects on fetal ketones or fetal lipid metabolism

In conclusion, insulin treatment of gestational diabetes may be more effective than metformin in ameliorating maternal dyslipidemia, although birth weight and other pregnancy outcomes were similar among the study groups. Our results suggest that the maternal metabolome could be helpful in identifying patients who benefit the most from metformin or insulin treatment. The long-term implications of elevated cord serum alanine merits further study.

KEYWORDS: Gestational diabetes, metformin, insulin, metabolism, metabolome, metabolomics, low-grade inflammation, birth weight

TURUN YLIOPISTO
Lääketieteellinen tiedekunta
Kliininen laitos
Synnytys- ja naistentautioppi ja sisätautioppi
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TIIVISTELMÄ

Raskausdiabetes on yleinen ongelma, joka lisää raskauden riskejä sekä ennustaa äidin ja lapsen myöhempää sairastavuutta. Raskausdiabetesta hoidetaan elintapamuutoksin sekä tarvittaessa lääkehoidolla. Korkean verensokerin lisäksi raskausdiabetekseen liittyy rasva- ja aminohappoaineenvaihdunnan sekä matala-asteisen tulehduksen häiriöitä. Toistaiseksi metformiinin vaikutuksia näihin muutoksiin insuliinihoitoon verrattuna ei kunnolla tunneta.

Tässä aiemman satunnaistetun tutkimuksen jatkoanalyysissa verrattiin raskausdiabeteksen metformiini- (n = 110) ja insuliinihoitojen (n = 107) vaikutuksia äidin aineenvaihdunnan molekyyleihin (metabolomiin), tulehdusmerkkiaineisiin ja insuliinin kaltaista kasvutekijää sitovaan proteiini 1:een. Lääkehoidon aloitusvaiheen vertailuun otetiin myös potilaita (n = 126), jotka eivät tarvinneet verenglukoosia alentavaa lääkitystä. Napanuoraverinäytteet otettiin synnytyksen jälkeen kaikissa kolmessa ryhmässä metformiinin vaikutusten tutkimiseksi.

Metformiinihoidetuilla äideillä seerumin alaniinin, triglyseridien kokonaismäärän, erittäin matalatiheyksisten lipoproteiinien triglyseridien sekä rasvahappojen kokonaismäärän pitoisuudet nousivat enemmän kuin insuliinihoidetuilla. Napaveren metabolomissa ainoastaan alaniini oli merkitsevästi korkeampi metformiiniryhmässä. Äidin verenkierrossa erityisesti erittäin matalatiheyksisen lipoproteiinin kolesteroli sekä apolipoproteiini B:n ja A-1:n suhde olivat yhteydessä korkeampaan syntymäpainoon ja nämä yhteydet olivat vahvempia metformiiniryhmässä. Napaveressä omega-6-rasvahapot liittyivät korkeampaan ja omega-3-rasvahapot matalampaan syntymäpainoon. Metformiinilla ei ollut vaikutuksia sikiön ketoneihin tai rasva-aineenvaihduntaan.

Raskausdiabeteksen insuliinihoito metformiiniin verrattuna saattaa olla tehokkaampi äidin rasva-aineenvaihdunnan muutosten lieventämisessä, vaikka syntymäpainoissa tai raskauskomplikaatioissa ei ollut eroja ryhmien välillä. Tulokset viittaavat siihen, että tulevaisuudessa äidin metabolomista voisi olla apua niiden potilaiden tunnistamisessa, jotka hyötyvät ensisijaisesti joko metformiini- tai insuliinihoidosta. Kohonneen napaveren alaniinin mahdolliset vaikutukset lasten myöhempään terveyteen vaativat lisätutkimuksia.

AVAINSANAT: Raskausdiabetes, metformiini, insuliini, aineenvaihdunta, metabolomi, metabolomiikka, aminohapot, matala-asteinen tulehdus, syntymäpaino

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Abbreviations

AA	amino acids
ALA	α-linolenic acid (18:3n-3)
AMPK	adenosine monophosphate-activated protein kinase
ANCOVA	analysis of covariance
ANOVA	analysis of variance
apoA-1	apolipoprotein A-1
apoB	apolipoprotein B
ARA	arachidonic acid (20:4n-6)
BCAA	branched-chain amino acids
BMI	body mass index
pBMI	pre-pregnancy body mass index
BW	birth weight
CETP	cholesterol ester transfer protein
CGM	continuous glucose monitoring
CI	confidence intervals
CRP	C-reactive protein
hsCRP	high-sensitivity C-reactive protein
DGLA	dihomo-γ-linolenic acid (20:3n-6)
DHA	docosahexaenoic acid (22:6n-3)
ELISA	enzyme-linked immunosorbent assay
EPA	eicosapentaenoic acid (20:5n-3)
FA	fatty acids
FDR	false discovery rate
FGR	fetal growth restriction
GDM	gestational diabetes
GLA	γ-linolenic acid (18:3n-6)
GlycA	glycoprotein acetylation
gw	gestational weeks
GWG	gestational weight gain
HAPO	Hyperglycemia and Adverse Pregnancy Outcomes (study)

HbA1c	glycated hemoglobin
HDL	high-density lipoprotein
IDL	intermediate-density lipoprotein
IADPSG	International Association of the Diabetes and Pregnancy Study Groups
IL-6	interleukin 6
IGFBP-1	insulin-like growth factor-binding protein 1
non-pIGFBP-1	non-phosphorylated IGFBP-1
low-pIGFBP-1	low-phosphorylated IGFBP-1
high-pIGFBP-1	high-phosphorylated IGFBP-1
IGF-1	insulin-like growth factor 1
IQR	interquartile range
I.V.	intravenous
LA	linoleic acid (18:2n-6)
LDL	low-density lipoprotein
LGA	large for gestational age
LPL	lipoprotein lipase
MMP-8	matrix metalloproteinase 8
MS	mass spectrometry
mTOR	mammalian target of rapamycin
MUFA	monounsaturated fatty acids
NDDG	National Diabetes Data Group
NEFA	non-esterified fatty acids
NICU	neonatal intensive care unit
NMR	nuclear magnetic resonance
NPH	neutral protamine Hagedorn
OGTT	oral glucose tolerance test
OR	odds ratio
PCA	principal component analysis
PCOS	polycystic ovary syndrome
PLS	partial least squares
PLS-DA	partial least squares discriminant analysis
PUFA	polyunsaturated fatty acids
RR	risk ratio
SD	standard deviation
SE	standard error
SGA	small for gestational age
TCA	tricarboxylic acid (cycle)
TG	triglycerides
TNF-α	tumor necrosis factor α

T1DM	type 1 diabetes
T2DM	type 2 diabetes
VLDL	very low-density lipoprotein

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Huhtala MS, Tertti K, Pellonperä O, Rönnemaa T. Amino acid profile in women with gestational diabetes mellitus treated with metformin or insulin. *Diabetes Research and Clinical Practice*, 2018;146:8–17.

II Huhtala MS, Tertti K, Juhila J, Sorsa T, Rönnemaa T. Metformin and insulin treatment of gestational diabetes: effects on inflammatory markers and IGF-binding protein-1 – secondary analysis of a randomized controlled trial. *BMC Pregnancy and Childbirth*, 2020;20(1):401.

III Huhtala MS, Tertti K, Rönnemaa T. Serum lipids and their association with birth weight in metformin and insulin treated patients with gestational diabetes. *Diabetes Research and Clinical Practice*, 2020;170:108456.

IV Huhtala MS, Rönnemaa T, Pellonperä O, Tertti K. Cord serum metabolome and birth weight in gestational diabetes patients treated with metformin, insulin, or diet alone. *Submitted*. 2021

1 Introduction

Gestational diabetes mellitus (GDM), defined as hyperglycemia with onset during pregnancy (American Diabetes Association, 2016; Committee on Practice Bulletins—Obstetrics, 2018), affects every fifth pregnancy in Finland (Finnish Institute of Health and Welfare, 2020). GDM poses both the mother and the fetus an increased risk of adverse pregnancy outcomes (Langer *et al.*, 2005a; HAPO Study Cooperative Research Group *et al.*, 2008). Besides an increased risk of short-term adverse outcomes, such as macrosomia, preeclampsia, cesarean delivery, neonatal hypoglycemia, hyperbilirubinemia and a need for neonatal intensive care (Langer *et al.*, 2005a; HAPO Study Cooperative Research Group *et al.*, 2008), GDM is associated with an increased risk of type 2 diabetes (T2DM) for the mother (Vounzoulaki *et al.*, 2020) and with obesity and metabolic syndrome for the offspring later in life (Clausen *et al.*, 2009; Lowe *et al.*, 2019).

With adequate treatment, short-term adverse events associated with GDM can be mitigated (Crowther *et al.*, 2005; Langer *et al.*, 2005a; Landon *et al.*, 2009). Treatment of GDM starts with medical nutritional therapy and lifestyle changes and if they alone prove insufficient for achieving the glycemic goals, insulin treatment is initiated (Working group established by the Finnish Medical Society Duodecim, 2013; Committee on Practice Bulletins—Obstetrics, 2018). Metformin has also been studied as an alternative for insulin in GDM (Rowan *et al.*, 2008; Ijäs *et al.*, 2011; Spaulonci *et al.*, 2013; Tertti *et al.*, 2013) and based on meta-analyses metformin may be even superior to insulin in terms of lesser gestational weight gain (GWG) and of a lower risk of large for gestational age (LGA) babies, neonatal hypoglycemia and hypertensive disorders (Butalia *et al.*, 2017; Farrar *et al.*, 2017b). In the long term, metformin exposure has however been related to increased offspring weight and body mass index (BMI) (van Weelden *et al.*, 2018; Hanem *et al.*, 2019), which has caused some concerns over the safety of metformin use in pregnancy.

Although maternal hyperglycemia is known to affect pregnancy outcomes (HAPO Study Cooperative Research Group *et al.*, 2008) and long-term offspring health (Clausen *et al.*, 2009; Lowe *et al.*, 2019), the underlying mechanisms are not well characterized. Recent data however suggests that maternal metabolome could be involved (Lowe *et al.*, 2014).

Metabolomics refers to the study of metabolites and it comprises identification and quantification of small molecules, like amino acids, lipids and intermediary metabolites of glucose, lipid and amino acid metabolism, i.e., the metabolome (Lowe *et al.*, 2014). GDM is associated with alterations in the maternal metabolome (Huynh *et al.*, 2014a; White *et al.*, 2017; Mokkala *et al.*, 2020b) and low-grade inflammation (Bao *et al.*, 2015; White *et al.*, 2017).

Maternal hyperglycemia is a significant contributor to fetal growth in GDM (HAPO Study Cooperative Research Group *et al.*, 2008), but there is also an evident, yet complex, interaction between the maternal metabolome and birth weight (BW) (Catalano *et al.*, 2011; Hellmuth *et al.*, 2019; Kadakia *et al.*, 2019a). Both the maternal metabolome (Chorell *et al.*, 2017) and the metabolome of the neonate (Standl *et al.*, 2014; Patel *et al.*, 2018) may predict future metabolic health of the offspring.

Metformin administration has been shown to alter the metabolome outside pregnancy (Cai *et al.*, 2009; Huo *et al.*, 2009; Zhang *et al.*, 2014; Irving *et al.*, 2015; Preiss *et al.*, 2016; Rotroff *et al.*, 2016; Eppinga *et al.*, 2017; Safai *et al.*, 2018). In GDM, metformin treatment, compared to insulin, increases the concentration of total plasma triglycerides (TG), but not of high-density lipoprotein (HDL) nor low-density lipoprotein (LDL) cholesterol (Barrett *et al.*, 2013a). However, the detailed effects of metformin on the metabolome in pregnancy have not been studied. Metformin has also been shown to ameliorate low-grade inflammation (Desai *et al.*, 2013; Goldberg *et al.*, 2014), but study results on this topic in pregnant subjects are inconclusive (Barrett *et al.*, 2013a; Wang *et al.*, 2017a).

The aim of this study was to characterize the effects of metformin in the treatment of GDM on the maternal serum metabolome and inflammatory markers and to compare these effects with the ones caused by insulin treatment. Another aim was to compare the maternal metabolome and inflammatory markers at the time of the diagnosis of GDM between patients who required pharmacological treatment and patients who achieved the glycemic targets with diet and lifestyle modifications alone. The specific aim was to identify metabolic markers associated with fetal growth and other perinatal outcomes and to assess whether these associations differed among treatment groups. In the last part of the study, the effects on the neonatal cord serum metabolome of metformin, compared to insulin and diet and lifestyle treatment of GDM alone were examined. Here, serum obtained from the neonatal cord was considered as a proxy of fetal metabolism.

2 Review of the Literature

2.1 Gestational diabetes

Gestational diabetes (GDM) has traditionally been defined as a hyperglycemia with onset or recognition during pregnancy (Alberti *et al.*, 1998; Committee on Practice Bulletins—Obstetrics, 2013). Newer narrower definitions only include hyperglycemia that has developed during pregnancy (American Diabetes Association, 2016; Committee on Practice Bulletins—Obstetrics, 2018), although the diagnosis is often not unambiguous. In Finland the national guidelines from 2013 (Working group established by the Finnish Medical Society Duodecim, 2013) define GDM as a state of abnormal glucose metabolism first diagnosed during pregnancy.

The incidence of GDM has risen steadily in Europe during the last 40 years (Eades *et al.*, 2017) and the global prevalence is expected to rise (Yuen *et al.*, 2019). The causes for this increasing prevalence are not completely understood, although the underlying factors probably include increased maternal age and obesity as well as changes in diagnostic criteria and screening penetration (Ferrara, 2007). Currently every fifth pregnancy in Finland is being complicated by GDM (Finnish Institute of Health and Welfare, 2020), but the prevalence varies across countries, depending on ethnicity, maternal age, screening and diagnostic criteria (Eades *et al.*, 2017).

2.1.1 Pathogenesis

Normal pregnancy is characterized by increased insulin resistance (Catalano *et al.*, 1991) and basal hepatic glucose production (Catalano *et al.*, 1992), coupled to an increase in the number of pancreatic β-cells (Butler *et al.*, 2010). Hyperglycemia ensues when the insulin secretion is insufficient to meet the requirements set by increased insulin resistance (Buchanan *et al.*, 2005).

Insulin resistance increases and β-cell function becomes impaired in GDM (Xiang *et al.*, 1999) and it has been proposed that the two abnormalities are interconnected and do not generally appear alone (Buchanan, 2001).

The causes for impaired glucose metabolism in pregnancy are not fully known. The following circumstances have been associated with GDM, but their relative importance is unclear: increased leptin (Kautzky-Willer *et al.*, 2001), tumor necrosis

factor α (TNF-α) (Kirwan *et al.*, 2002), C-reactive protein (CRP) (Wolf *et al.*, 2003) and interleukin 6 (IL-6) (Kuzmicki *et al.*, 2009), decreased adiponectin (Retnakaran *et al.*, 2004) and metabolic aberrations, e.g., increased fatty acids (FA) and branched-chain amino acids (BCAA) (Huynh *et al.*, 2014b; Scholtens *et al.*, 2014).

Along with impaired insulin resistance (Catalano *et al.*, 1993, 1999), metabolic aberrations are observed in women already before the development of GDM (Bentley-Lewis *et al.*, 2015; White *et al.*, 2017; Mokkala *et al.*, 2020c). Whether these changes are causally related to GDM is not known.

Monogenic diabetes (MODY) (Gjesing *et al.*, 2017; Zubkova *et al.*, 2019), or β-cell antibodies predicting autoimmune diabetes (type 1 diabetes, T1DM) (Nilsson *et al.*, 2007) may be found in a small fraction of patients in whom hyperglycemia is diagnosed in pregnancy. Still, for most patients the origin of GDM is apparently multifactorial and heterogeneous.

2.1.2 Diagnosis and risk factors

GDM is diagnosed using either the two-hour or three-hour oral glucose tolerance test (OGTT), but thus far there is no global consensus regarding the ultimate diagnostic criteria or screening algorithm. The first OGTT criteria by O'Sullivan *et al.* published in 1964, were defined as two standard deviations (SD) above the mean glucose values for each of the measurements in the three-hour OGTT (O'Sullivan *et al.*, 1964). These criteria were later translated into plasma glucose values (rather than whole blood glucose, as in the original publication) and adopted into the National Diabetes Data Group (NDDG) guidelines (National Diabetes Data Group, 1979). In 1982, the criteria of Carpenter and Coustan were published and they presented lower thresholds than the O'Sullivan criteria (Carpenter *et al.*, 1982). The aim of all of these criteria was to identify the mothers who were at risk of developing diabetes after pregnancy rather than to identify those at risk of adverse perinatal outcomes (Metzger *et al.*, 2007).

To address this limitation, a large prospective multicenter study, the Hyperglycemia and Adverse Pregnancy Outcomes (HAPO) study, was conducted (HAPO Study Cooperative Research Group *et al.*, 2008). This study showed that there are no discrete threshold values but rather a linear association between maternal glucose levels and four predefined pregnancy outcomes: BW above the 90th percentile for gestational age, primary cesarean delivery, clinically diagnosed neonatal hypoglycemia and cord-blood serum C-peptide level above the 90th percentile.

Based on the HAPO results, International Association of the Diabetes and Pregnancy Study Groups (IADPSG) published the first diagnostic cut-off values that took short-term pregnancy outcomes into account (International Association of

Diabetes and Pregnancy Study Groups Consensus Panel *et al.*, 2010). These criteria are nonetheless based on observational data and, while randomized trials are awaited, current evidence is insufficient to support superiority of one screening method over another (Farrar *et al.*, 2017a).

The NDDG, Carpenter & Coustan, IADPSG and Finnish national OGTT criteria are summarized in Table 1.

Table 1. Oral glucose tolerance test threshold values according to different criteria.

	NDDG	**Carpenter & Coustan**	**IADPSG**	**Finnish national criteria**
Year	*1979*	*1982*	*2010*	*2008, 2013*
Screening test	50 g GCT	50 g GCT	-	-
Diagnostic test	100 g 3-hour OGTT	100 g 3-hour OGTT	75 g 2-hour OGTT	75 g 2-hour OGTT
OGTT thresholds Plasma glucose (mmol/l)				
Fasting	5.8	5.3	5.1	5.3
One hour	10.6	10.0	10.0	10.0
Two hours	9.2	8.6	8.5	8.6
Three hours	8.0	7.8	-	-
No. of increased values required for diagnosis	2	2	1	1

NDDG: National Diabetes Data Group, IADPSG: International Association of Diabetes and Pregnancy Study Groups, GCT: glucose challenge test, OGTT: oral glucose tolerance test. Adapted from (National Diabetes Data Group, 1979; Carpenter *et al.*, 1982; International Association of Diabetes and Pregnancy Study Groups Consensus Panel *et al.*, 2010; Working group established by the Finnish Medical Society Duodecim, 2013).

Several known risk factors for GDM have been established. In a recent umbrella review of meta-analyses, obesity and hypothyroidism were the risk factors with the most convincing evidence (Giannakou *et al.*, 2019). Overweight, snoring, sleep-disordered breathing, polycystic ovary syndrome and a family history of diabetes were also "highly suggestive" of being associated with an increased GDM risk (Giannakou *et al.*, 2019). An alternative for universal screening of GDM is risk factor based screening, but whether any screening strategy ultimately leads to improved perinatal outcomes cannot be judged by the available evidence (Tieu *et al.*, 2017).

The Finnish national guidelines published 2008 and revised 2013 endorse universal screening for GDM (Working group established by the Finnish Medical Society Duodecim, 2013). A two-hour 75 g OGTT is recommended for every

pregnant woman at 24–28 gestational weeks (gw), except for nulliparous women aged < 25 years and BMI < 25 kg/m^2 and parous women aged < 40 years, BMI < 25 kg/m^2 and no previous GDM or macrosomia. An additional early OGTT at 12–16 gw is recommended for subjects at high risk.

2.1.3 Treatment

The treatment of GDM leads to an unequivocal reduction in adverse perinatal outcomes (Crowther *et al.*, 2005; Langer *et al.*, 2005a; Landon *et al.*, 2009). A systematic review and meta-analysis of GDM treatment by the U.S. Preventive Services Task Force showed reduced risks of preeclampsia (risk ratio, RR: 0.62; confidence intervals, CI: 0.43, 0.89), shoulder dystocia (RR: 0.42; CI: 0.23, 0.77) and macrosomia (RR: 0.50; CI: 0.35, 0.71) (Hartling *et al.*, 2013). The treatment did not affect maternal GWG, the risk of birth injury, neonatal hypoglycemia, cesarean delivery, induction of labor, small for gestational age (SGA), nor neonatal intensive care unit (NICU) admission (Hartling *et al.*, 2013).

Generally, patients diagnosed with GDM are provided diet and lifestyle counselling and advised to self-monitor fasting and postprandial glucose values, although the optimal glucose targets in GDM are not known (Prutsky *et al.*, 2013; Crowther *et al.*, 2018; Popova *et al.*, 2020). The Finnish guidelines recommend initiation of pharmacological treatment if the glucose targets for fasting glucose < 5.5 mmol/l or 1 h postprandial glucose < 7.8 mmol are not met despite diet and lifestyle modifications (Working group established by the Finnish Medical Society Duodecim, 2013).

Insulin therapy is usually begun with neutral protamine Hagedorn (NPH) insulin (Working group established by the Finnish Medical Society Duodecim, 2013; Committee on Practice Bulletins—Obstetrics, 2018). Rapid-acting insulin lispro and insulin aspart may be used either alone or in combination with NPH insulin if the postprandial glucose values tend to remain elevated.

Although insulin has been the standard pharmacological treatment, also metformin and glyburide have been studied and may be used to treat GDM (Langer *et al.*, 2000; Rowan *et al.*, 2008; Farrar *et al.*, 2017b). Compared to injectable insulin oral antidiabetic drugs are tempting due to lower price and ease of administration. Metformin treatment of GDM is currently accepted as an alternative for insulin by Finnish guidelines and will be reviewed later in detail. Glyburide crosses the placenta (Hebert *et al.*, 2009) and is associated with an increased risk of neonatal hypoglycemia (Farrar *et al.*, 2017b; Song *et al.*, 2017) and is possibly less efficient in reducing the risk of macrosomia (Farrar *et al.*, 2017b; Song *et al.*, 2017). The use of glyburide is not supported by the Finnish guidelines (Working group established by the Finnish Medical Society Duodecim, 2013).

2.1.4 Consequences of gestational diabetes

Perinatal and neonatal outcomes

Adverse pregnancy outcomes are more common in pregnancies complicated by GDM than in normal pregnancies (Langer *et al.*, 2005a). There is a linear association between, on one hand, maternal glycemia and high BW (HAPO Study Cooperative Research Group *et al.*, 2008) and, on the other hand, the risk of LGA infants and macrosomia in GDM (Langer *et al.*, 2005a; O'Sullivan *et al.*, 2011). The risk of shoulder dystocia is increased not only by the higher BW of the neonate, but the maternal diabetes acts also as an independent risk factor (Nesbitt *et al.*, 1998), probably through altered anthropometrics of the infants exposed to hyperglycemia in utero. Hence, cesarean delivery should be considered if the estimated fetal weight exceeds 4500 g in mothers with GDM (Working group established by the Finnish Medical Society Duodecim, 2013; Committee on Practice Bulletins—Obstetrics, 2018).

The overall risk of cesarean delivery is also higher in GDM compared to normal pregnancies (Langer *et al.*, 2005a; HAPO Study Cooperative Research Group *et al.*, 2008; O'Sullivan *et al.*, 2011). Retrospective data indicate that the induction of labor near term at 38+0 – 39+6 gw may be associated with reduced need for cesarean deliveries (Melamed *et al.*, 2016).

GDM and preeclampsia share common risk factors and GDM is, not surprisingly, associated with an increased risk of preeclampsia. When adjusted for C-peptide, the association between maternal glycemia and incidence of preeclampsia was attenuated in the HAPO study population, suggesting that insulin resistance may play a significant role in the development of preeclampsia (Hyperglycemia and Adverse Pregnancy Outcome (HAPO) Study Cooperative Research Group, 2010). Adequate treatment of GDM may lower the risk of preeclampsia (Hartling *et al.*, 2013).

The neonatal complications of GDM include neonatal hypoglycemia, respiratory complications, hyperbilirubinemia and an increased overall risk of NICU admission (Langer *et al.*, 2005a; O'Sullivan *et al.*, 2011). The increased rates of neonatal hypoglycemia in the presence of maternal diabetes is thought to be due to neonatal hyperinsulinism (Pedersen *et al.*, 1954) and, correspondingly, maternal glycemia during the late 2nd and early 3rd trimester is related to risk on neonatal hypoglycemia (HAPO Study Cooperative Research Group *et al.*, 2008). In T1DM modest improvements in glycemic control may decrease the risk of neonatal hypoglycemia (Yamamoto *et al.*, 2019), but these results might not be applicable in GDM (Hartling *et al.*, 2013; Popova *et al.*, 2020). Furthermore, glyburide treatment of GDM may even increase the risk of neonatal hypoglycemia (Farrar *et al.*, 2017b; Song *et al.*, 2017).

Fetal hyperinsulinemia has opposing effects to cortisol on lung maturation and explains the delayed lung maturation of fetuses in GDM (Gluck *et al.*, 1973). In a large retrospective cohort GDM that required insulin treatment was (adjusted OR: 1.7; CI: 1.4, 2.1), while diet controlled GDM was not (adjusted OR: 1.1; CI: 0.9, 1.3) associated with respiratory distress (Billionnet *et al.*, 2017). There is no evidence that treatment of GDM would reduce the risk of neonatal respiratory complications (Hartling *et al.*, 2013).

Neonates born to mothers with diabetes have an increased risk of hyperbilirubinemia due to a larger red cell mass, ineffective erythropoiesis and immature bilirubin conjugation (Nold *et al.*, 2004). Thus, the glycated hemoglobin (HbA1c) values in T1DM measured near delivery are associated with the neonatal hematocrit (Green *et al.*, 1992) and with the level of amniotic fluid erythropoietin (Teramo *et al.*, 2004). Treatment of GDM may lower the rates of neonatal hyperbilirubinemia (Hartling *et al.*, 2013).

Long-term outcomes

Women who have had GDM in a previous pregnancy are at increased risk of GDM in subsequent pregnancies (Getahun *et al.*, 2010), as well as of T2DM later in life compared to women with normoglycemic pregnancies (RR: 9.51; CI: 7.14, 12.67) (Vounzoulaki *et al.*, 2020). Whether this risk can be reduced was studied as a part of a larger Diabetes Prevention Program study. Women with prior GDM were randomized to intensive lifestyle intervention, metformin or placebo treatment with the main outcome to prevent T2DM (Ratner *et al.*, 2008). Treatment lasted for 3 years and both lifestyle and metformin treatment roughly halved the risk of T2DM compared to placebo. In the 10-year follow-up study the difference was sustained (Aroda *et al.*, 2015). Based on this data the numbers needed to treat to prevent one case of T2DM in 10 years was 7.2 for metformin and 11.3 for lifestyle interventions (Aroda *et al.*, 2015). Similar results were reported for lifestyle intervention in the Finnish Diabetes Prevention Study which targeted subjects with impaired glucose tolerance (Tuomilehto *et al.*, 2001). Not only the risk of T2DM but also the risk of hypertension and cardiovascular disease is increased in women with a history of GDM, independent of confounding factors (Pirkola *et al.*, 2010; Tobias *et al.*, 2011; Kramer *et al.*, 2019).

Unfortunately, the long-term consequences of GDM are of concern not only to the mother but also to the neonate exposed to hyperglycemia in utero. In the early 1980's there were discussions that metabolic perturbations during pregnancy could affect fetal development also after the phase of organogenesis (Freinkel, 1980). Initial studies in Pima Indians found that fetal exposure to maternal hyperglycemia had long lasting consequences on the weight centiles and plasma glucose values of

the offspring (Petitt *et al.*, 1985). This effect was not fully explained by genetic susceptibility, as demonstrated in a study comparing siblings born either before or after the mother was diagnosed with T2DM (Dabelea *et al.*, 2000). Similar associations between maternal diabetes and adverse metabolic traits in the offspring were later confirmed in Danish and Finnish cohort studies (Clausen *et al.*, 2009; Kaseva *et al.*, 2019) and the HAPO follow-up study (Lowe *et al.*, 2019). Maternal GDM is associated with obesity, the metabolic syndrome and impaired glucose tolerance and insulin sensitivity in the offspring at 10–20 years of age (Clausen *et al.*, 2009; Lowe *et al.*, 2018, 2019).

When the neonates who have been exposed to a hyperglycemic environment in utero grow older, they are more likely to be overweight and have unfavorable metabolic traits than those not exposed. And when these subjects become pregnant they have an elevated risk of GDM, which creates an intergenerational vicious cycle of diabetes (Dabelea, 2007).

To facilitate lifestyle interventions and early recognition of pre-diabetes, follow-up of patients with GDM is recommended postpartum (Working group established by the Finnish Medical Society Duodecim, 2013; Committee on Practice Bulletins—Obstetrics, 2018).

2.1.5 Metabolic changes in gestational diabetes

Glucose metabolism

Maternal hyperglycemia is the hallmark of GDM. It is due to inadequate insulin secretion in the face of increased insulin resistance. In GDM, the inhibition of endogenous glucose production by insulin is reduced in both normal weight (Catalano *et al.*, 1993) and obese (Catalano *et al.*, 1999) subjects. Glucose clearance is also decreased in GDM (Xiang *et al.*, 1999).

Insulin sensitivity decreases as the pregnancy progresses (Catalano *et al.*, 1991). Longitudinal studies by Catalano *et al.* show that insulin sensitivity is impaired already before pregnancy in subjects who will subsequently be diagnosed with GDM (Catalano *et al.*, 1993, 1999). To compensate for the impaired insulin sensitivity, the first-phase and second-phase insulin responses increase from the pre-gravid state to early and late pregnancy (Catalano *et al.*, 1993, 1999). In lean subjects, the first-phase response during early and late pregnancy is attenuated in GDM (Catalano *et al.*, 1993), while in obese subjects there is no differences between GDM and non-GDM subjects regarding first-phase insulin secretion but the second-phase response is amplified already before pregnancy (Catalano *et al.*, 1999). GDM patients are heterogeneous with respect to insulin secretion and insulin effects. When GDM patients in a prospective cohort were divided into three subclasses; predominantly

defective insulin secretion, predominantly impaired insulin sensitivity and a mixed phenotype, the risk of pregnancy complications was highest in the impaired insulin sensitivity group (Powe *et al.*, 2016).

In the physiological, non-pregnant state, insulin regulates non-esterified FA (NEFA) concentrations by promoting their uptake and by inhibiting their release by lipolysis in the adipose tissue. In GDM, however, the regulation NEFA metabolism is also defective. NEFA concentrations correlate strongly with glucose production (Xiang *et al.*, 1999) and may promote insulin resistance (Sivan *et al.*, 1998).

In GDM, utilization of ketogenic amino acids, BCAA and NEFA as an energy source seems to raise the concentrations of maternal ketones (Pappa *et al.*, 2007; Dudzik *et al.*, 2014), and increased ketones (including 3-hydroxybutyrate) are a common finding in GDM in contrast to pregnancies with normal glucose tolerance (Montelongo *et al.*, 1992; Pappa *et al.*, 2007; Scholtens *et al.*, 2014; Dudzik *et al.*, 2017; Mokkala *et al.*, 2020b). Ketones cross the placenta but whether they affect fetal development and growth is not known.

Amino acids

Table 2. List of twenty amino acids common in human metabolism.

Other amino acids	Branched-chain amino acids	Aromatic amino acids
Alanine #	Isoleucine *	Phenylalanine *
Arginine	Leucine *	Tyrosine
Asparagine	Valine *	Histidine *
Aspartic acid		Tryptophan *
Cysteine		
Glutamic acid		
Glutamine #		
Glycine		
Lysine *		
Methionine *		
Proline		
Serine		
Threonine *		

* Essential amino acids, # Most important glucogenic amino acids

Besides glucose, the metabolism of most other metabolites, including amino acids (Table 2), is altered in GDM. In normal pregnancy, the serum concentration of alanine, phenylalanine and histidine increase while the concentrations of glutamine, glycine, valine and tyrosine decrease (Wang *et al.*, 2016). Compared to the first trimester, glutamic acid and threonine are higher in the third trimester (Lindsay *et al.*, 2015). The BCAA leucine and valine, like arginine, glycine, phenylalanine, tryptophan, serine and tyrosine, decrease as pregnancy proceeds (Lindsay *et al.*, 2015). Early studies on GDM demonstrated altered amino acid concentrations. Particularly plasma BCAA were increased in GDM (Metzger *et al.*, 1980), although most of the studies were small and the results heterogeneous (Metzger *et al.*, 1980; Butte *et al.*, 1999; Cetin *et al.*, 2005; Pappa *et al.*, 2007).

In two large contemporary prospective studies on overweight and obese pregnant women, the concentration of the BCAA isoleucine and leucine, and of phenylalanine were higher in pregnancies complicated by GDM, both before and after the diagnosis of GDM (White *et al.*, 2017; Mokkala *et al.*, 2020b, 2020c). Of note, all subjects in these studies were overweight or obese and the women with GDM were usually managed without pharmacological treatment (Poston *et al.*, 2015; Mokkala *et al.*, 2020b). Maternal alanine may be higher prior to the GDM diagnosis (Bentley-Lewis *et al.*, 2015; Mokkala *et al.*, 2020c). The metabolome at mean 28 gw was compared between low fasting-plasma glucose (< 10^{th} centile) and high fasting-plasma glucose (> 90^{th} centile) groups of mothers of Northern European ancestry in the HAPO study (Scholtens *et al.*, 2014). In this cohort, high fasting plasma glucose was associated with higher levels of isoleucine, proline, valine, alanine, leucine, serine, ornithine, glutamic acid and threonine. The association between increased levels of arginine during the first (Nevalainen *et al.*, 2016) and third (Rahimi *et al.*, 2017) trimester and GDM have been confirmed in later studies.

In pregnancy, the serum levels of several maternal amino acids, including alanine, leucine/isoleucine[1], phenylalanine and proline, are positively associated with OGTT 1 h glucose levels (Scholtens *et al.*, 2016) and inversely related to insulin sensitivity (Sandler *et al.*, 2017). The BCAA degradation pathway in particular, was related to insulin sensitivity (Sandler *et al.*, 2017).

In conclusion, GDM seems to be associated with increased maternal serum levels of alanine and BCAA. In T2DM, BCAA have been related to increased insulin resistance (Guasch-Ferré *et al.*, 2016) and, based on data from large genetic studies, BCAA lie on a causal pathway from insulin resistance to frank T2DM (Lotta *et al.*, 2016; Wang *et al.*, 2017b). In GDM, compared to normal pregnancies, BCAA stay

[1] The analysis method used does not differentiate between leucine and isoleucine.

elevated postpartum (Chorell *et al.*, 2017) and are associated with an increased risk of T2DM (Andersson-Hall *et al.*, 2018).

Lipids

Increased insulin resistance and increased estrogen concentrations cause apparent alterations in lipid metabolism in pregnancy (Figure 1) (reviewed in (Herrera *et al.*, 2016)). Hepatic production of very low-density lipoprotein (VLDL) is increased during pregnancy, due to increased estrogen effects, maternal energy intake and insulin resistance (Alvarez *et al.*, 1996; Herrera *et al.*, 2016). Simultaneously, increased estrogen effects and insulin resistance downregulate lipoprotein lipase (LPL) (Herrera *et al.*, 1988; Mead *et al.*, 2002) and hepatic lipase (Alvarez *et al.*, 1996), which results in accumulation of VLDL (Jimenez *et al.*, 1988; Wang *et al.*, 2016). TG are further transferred from VLDL to LDL and HDL by cholesterol ester transfer protein (CETP), the activity of which is increased by enhanced estrogen activity during pregnancy (Silliman *et al.*, 1993; Iglesias *et al.*, 1994). This increases TG also in LDL and HDL particles (Wang *et al.*, 2016).

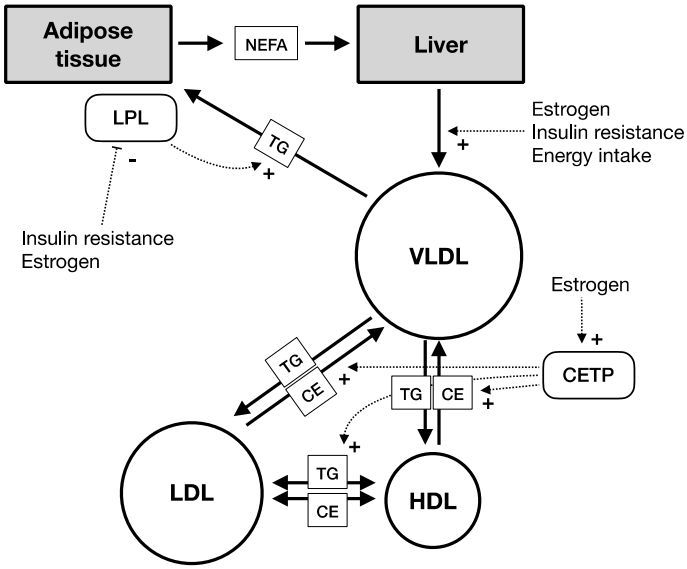

Figure 1. Major changes in lipid metabolism in pregnancy. VLDL: very low-density lipoprotein, LDL: low-density lipoprotein, HDL: high-density lipoprotein, TG: triglycerides, CE: cholesterol esters, NEFA: non-esterified fatty acids, CETP: cholesterol ester transfer protein, LPL: lipoprotein lipase. Modified from a review by Herrera et al. (2016).

There is ample evidence regarding pronounced dyslipidemia in GDM compared to normal pregnancy. Although increased in normal pregnancy, VLDL particle

concentrations are even higher in patients with GDM already before the diagnosis (White *et al.*, 2017; Mokkala *et al.*, 2020c, 2020b). Interestingly, also small HDL particle concentrations are higher in GDM than normal pregnancies (White *et al.*, 2017; Mokkala *et al.*, 2020c). Based on a meta-analysis, serum TG in all trimesters is higher in patients with GDM (Ryckman *et al.*, 2015) and, according to data from two prospective studies, this difference is evident in most lipoprotein subclasses (White *et al.*, 2017; Mokkala *et al.*, 2020c, 2020b). GDM is not associated with alterations in serum total cholesterol (Ryckman *et al.*, 2015; White *et al.*, 2017; Mokkala *et al.*, 2020b), but when divided into lipoprotein subclasses HDL cholesterol is lower in GDM throughout pregnancy (Ryckman *et al.*, 2015). The results from more detailed lipoprotein subclass analyses have been contradictory: Mokkala *et al.* found cholesterol in large HDL to be decreased and cholesterol in medium and small HDL to be increased before GDM was diagnosed (Mokkala *et al.*, 2020c), while White *et al.* reported non-significant reductions in all subclasses (White *et al.*, 2017). On the other hand, White *et al.* reported that, in the third trimester, HDL in most of the subclasses is decreased, while Mokkala *et al.* reported no differences (White *et al.*, 2017; Mokkala *et al.*, 2020b). First-trimester LDL cholesterol may be higher in patients with GDM (Ryckman *et al.*, 2015), although this finding was not confirmed in first trimester samples of overweight and obese subjects taken prior to the diagnosis of GDM (White *et al.*, 2017; Mokkala *et al.*, 2020c). Pregnancy alone leads to TG enrichment of LDL and HDL particles (Montelongo *et al.*, 1992) and this phenomenon is further amplified by GDM (Mokkala *et al.*, 2020b).

Table 3. Selected omega-3 and omega-6 fatty acids important for human metabolism.

Omega-3 fatty acids		**Omega-6 fatty acids**	
ALA	18:3n-3 *α*-linolenic acid*	LA	18:2n-6 linoleic acid*
EPA	20:5n-3 eicosapentaenoic acid	GLA	18:3n-6 *γ*-linolenic acid
DHA	22:6n-3 docosahexaenoic acid	DGLA	20:3n-6 dihomo-*γ*-linolenic acid
		ARA	20:4n-6 arachidonic acid

* Essential fatty acids

There are two essential fatty acids, *α*-linolenic acid (ALA) and linoleic acid (LA) that need to be acquired from the diet, since they cannot be synthesized in human cells. There are also several omega-3 and omega-6 FA that are important for human metabolism (Table 3).

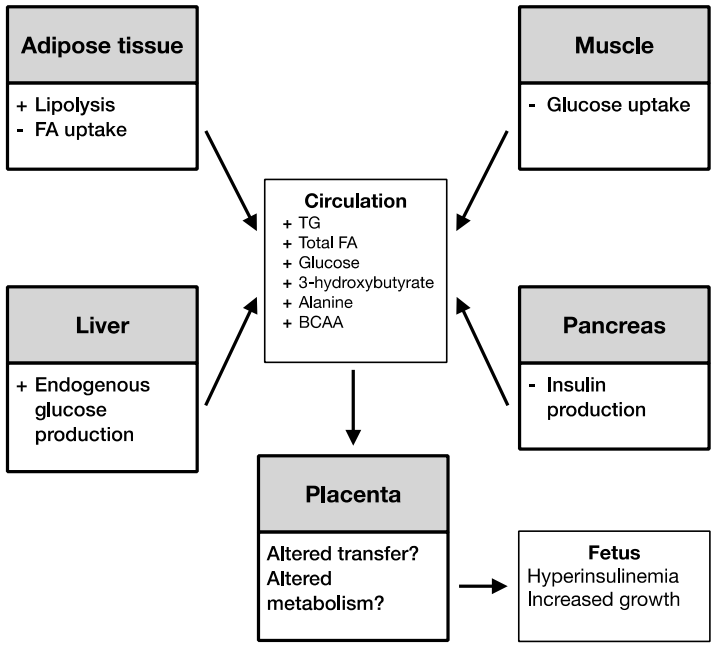

Figure 2. GDM is related to increased insulin resistance in the liver, adipose tissue and muscle and impaired insulin production in the pancreas (Catalano *et al.*, 1993, 1999; Friedman *et al.*, 1999; Xiang *et al.*, 1999). Consequently, impaired insulin mediated suppression of endogenous glucose production in the liver and reduced glucose uptake in the muscle leads to elevated plasma glucose values. In the liver insulin resistance leads to increased very low-density lipoprotein production and in the adipose tissue decreased fatty acid (FA) uptake and increased lipolysis (Alvarez *et al.*, 1996; Herrera *et al.*, 2016). Therefore circulating TG and FA are elevated in gestational diabetes (Montelongo *et al.*, 1992; Ryckman *et al.*, 2015). Reduced glucose utilization due to insulin resistance and increased lipolysis leads to increased ketogenesis (Pappa *et al.*, 2007; Dudzik *et al.*, 2014). The placental metabolism and transfer of metabolites may be altered in GDM (Desoye, 2018). Furthermore, these metabolites serve as fuel for the fetal growth, but may also promote fetal insulin secretion and consequently accelerated fetal growth (Freinkel, 1980; Catalano *et al.*, 2011). BCAA: branched-chain amino acids.

Serum total FA increase during pregnancy (Wang *et al.*, 2016). The changes are more pronounced in the third trimester and are similar for SFA, monounsaturated FA (MUFA) and PUFA (Wang *et al.*, 2016). Furthermore, the proportion of PUFA of all FA decreases and the proportions of MUFA and SFA of all FA increase in pregnancy (Wang *et al.*, 2016). The ratios of omega-6 FA and LA to total FA are lowered in pregnancy, while the effect on the corresponding omega-3 FA and docosahexaenoic acid (DHA) ratios is not as clear (Wang *et al.*, 2016). The decrease in the PUFA to total FA ratio and the increase in the MUFA to total FA ratio were augmented in GDM (White *et al.*, 2017; Mokkala *et al.*, 2020b). The ratio of LA and omega-6 FA to total FA is decreased in GDM (White *et al.*, 2017; Mokkala *et al.*,

2020b). However, several maternal SFA, MUFA and PUFA at term were slightly lower in GDM than normal pregnancies (Ortega-Senovilla *et al.*, 2020).

NEFA do not increase during pregnancy (Montelongo *et al.*, 1992; Lindsay *et al.*, 2015). But in GDM NEFA are higher already during the first trimester (Montelongo *et al.*, 1992) and may promote insulin resistance (Sivan *et al.*, 1998).

To summarize, GDM is associated with lower HDL cholesterol and higher VLDL lipoprotein concentrations and TG enrichment of maternal lipoproteins. Serum total TG and total FA are higher in GDM and the relative amounts of SFA, and MUFA are increased, while PUFA are decreased.

The metabolic alterations in GDM are summarized in Figure 2.

2.1.6 Low-grade inflammation in gestational diabetes

During pregnancy the maternal immune system undergoes changes that are considered essential for continued pregnancy (reviewed in (Trowsdale *et al.*, 2006)). Several inflammatory markers, including high-sensitivity CRP (hsCRP), glycoprotein acetylation (GlycA), IL-6 and TNF-α, increase during pregnancy (Christian *et al.*, 2014; Wang *et al.*, 2016). Maternal obesity has also a profound effect on inflammatory markers (Christian *et al.*, 2014), and dysregulation of the immune system has been associated with morbidities, such as GDM.

CRP is an acute-phase protein produced by the liver and widely used as a clinical marker of inflammation. High CRP in early pregnancy has been claimed to predict GDM (Wolf *et al.*, 2003), but this association is moderated when adjusted for BMI. In later studies, CRP has not been associated with a risk of GDM in overweight and obese women (White *et al.*, 2017), or in women with other GDM risk factors (Corcoran *et al.*, 2018). In the large HAPO cohort, CRP was associated with fasting, 1-h and 2-h values of maternal glucose at OGTT, also after adjustment for maternal BMI and C-peptide (Lowe *et al.*, 2010), but in another large cohort of overweight and obese women, CRP was not significantly elevated in GDM at the time of diagnosis (White *et al.*, 2017). One study reported an association between maternal CRP and GDM after adjustment for BMI at the end of the third trimester, but not at the second trimester (Leipold *et al.*, 2005). The CRP value after GDM has been diagnosed does predict persistence of impaired glucose tolerance post-partum (Durnwald *et al.*, 2018). In a study combining data from normal pregnancies and pregnancies complicated by impaired glucose tolerance, GDM or maternal overweight, CRP was associated with maternal BMI but an independent association with GDM was not confirmed (Retnakaran *et al.*, 2003).

IL-6 is a pro-inflammatory cytokine that is secreted by macrophages and adipocytes; secretion of acute phase proteins, such as CRP, in the liver is promoted by IL-6. However, IL-6 has also important anti-inflammatory properties, as

demonstrated in IL-6-deficient mice (Matthews *et al.*, 2010). During pregnancy, serum IL-6 increases in normal-weight subjects, but this rise is more subtle and the overall concentrations remain higher throughout pregnancy and postpartum in overweight and obese women (Stewart *et al.*, 2007; Friis *et al.*, 2013; Christian *et al.*, 2014). IL-6 in early pregnancy does not seem to be predictive for GDM (Bao *et al.*, 2015; White *et al.*, 2017) and studies after GDM has been diagnosed have provided diverse results (Georgiou *et al.*, 2008; Kuzmicki *et al.*, 2008; Morisset *et al.*, 2011; Özyer *et al.*, 2014; Ramirez *et al.*, 2014). Some studies have reported increased IL-6 levels in GDM, but they have been rather small and maternal BMI was higher in the women with GDM (Kuzmicki *et al.*, 2008; Morisset *et al.*, 2011). When groups similar regarding maternal BMI were compared or adjustment for BMI was done, there was no difference in IL-6 between women with and without GDM (Georgiou *et al.*, 2008; Özyer *et al.*, 2014; Ramirez *et al.*, 2014; White *et al.*, 2017). IL-6 polymorphism (rs1800795) is not associated with the occurrence of GDM (Feng *et al.*, 2018).

TNF-*α* is another proinflammatory cytokine secreted by adipose tissue and the placenta (Kirwan *et al.*, 2002). During pregnancy its concentration increases and is higher in overweight and obese subjects than non-obese subjects (Winkler *et al.*, 2002; Christian *et al.*, 2014). Two early studies reported increased TNF-*α* in GDM (Winkler *et al.*, 2002; Kinalski *et al.*, 2005), but this finding was not confirmed in a matched case-control study (Georgiou *et al.*, 2008) nor in a study on obese women (Ramirez *et al.*, 2014). In a systematic review TNF-*α* did not predict GDM, although the number of studies was small (Bao *et al.*, 2015).

GlycA is a more recent composite marker of inflammation, consisting mainly of *α*-1-acid glycoprotein, haptoglobin, *α*-1-antitrypsin, *α*-1-antichymotrypsin and transferrin (Bell *et al.*, 1987). It is assessed by nuclear magnetic resonance spectroscopy (NMR). Based on a large data set of over 26,000 initially healthy women in the Women's Health Study, GlycA predicted T2DM (Akinkuolie *et al.*, 2015) and cardiovascular events (Akinkuolie *et al.*, 2014). GlycA is also related to insulin resistance (Wang *et al.*, 2017b). During pregnancy GlycA increases (Wang *et al.*, 2016), although the concentration of the individual components of GlycA (including *α*-1 acid glycoprotein, *α*-1-antitrypsin and haptoglobin) have their own concentration-versus-time trajectories in pregnancy (Larsson *et al.*, 2008). In two large studies GlycA was increased in GDM after diagnosis as well as in early pregnancy prior to GDM diagnosis (White *et al.*, 2017; Mokkala *et al.*, 2020c, 2020b).

Matrix metalloproteinase 8 (MMP-8) is a collagenase involved in the breakdown of the extracellular matrix, but it is also associated with low-grade inflammation. In the non-pregnant population MMP-8 was increased in obese individuals and among smokers (Lauhio *et al.*, 2016) and it has been associated with cardiovascular

morbidity (Kormi *et al.*, 2017). In pregnancy MMP-8 in the cervix is related to cervical ripening (Kruit *et al.*, 2018) and in amniotic fluid with chorioamnionitis (Kim *et al.*, 2015). In one rather small study serum MMP-8 was positively associated with GDM (Akcalı *et al.*, 2017).

In conclusion, these inflammatory markers have a role in GDM and obesity-related insulin resistance, although there seems to be considerable overlap in serum concentrations of various markers between the GDM and non-GDM subjects.

2.2 Metformin

Metformin is an antidiabetic drug of the class of biguanides and was first synthesized from guanidine in 1922 (Werner *et al.*, 1922). Guanidine is derived from the *Galega officinalis* plant, also known as Goat's rue, or French lilac used in herbal remedies during the Middle Ages (Bailey, 2017).

In the 1950's, metformin therapy was first suggested for treating diabetes (Bailey, 2017). Other biguanides similar to metformin (phenformin and buformin) were associated with a significant risk of lactic acidosis (Luft *et al.*, 1978) and were withdrawn from the market. This led to drawbacks also in metformin use, although less so in Europe than elsewhere.

The research on metformin continued, nevertheless, and after landmark studies demonstrated the efficacy (DeFronzo *et al.*, 1995) and cardiovascular benefits of metformin (UK Prospective Diabetes Study (UKPDS) Group, 1998) metformin has become the mainstay therapy in T2DM.

Although metformin has been used for decades and almost one hundred years has passed since it was first synthesized, we still do not know exactly how metformin works (reviewed in (Rena *et al.*, 2017)).

On the molecular basis, metformin inhibits Complex I of the respiratory chain in the mitochondria, which reduces adenosine triphosphate production (Rena *et al.*, 2017). Metformin affects also the central cell signaling pathways including adenosine monophosphate-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) (Rena *et al.*, 2017).

Metformin is mostly absorbed in the small intestine where it accumulates in the mucosa resulting in concentrations magnitudes higher than in plasma (Bailey *et al.*, 2008). A positron emission tomography study with [^{11}C]metformin tracer showed that metformin accumulates fast in the liver and a more gradual increase was seen into muscle (Gormsen *et al.*, 2016). Metformin is eliminated unmetabolized via the kidneys.

Metformin is thought to ameliorate hyperglycemia by affecting the liver (Rena *et al.*, 2017). Metformin has been shown to reduce gluconeogenesis (Hundal *et al.*, 2000) and to inhibit the effects of glucagon (Miller *et al.*, 2013) in the liver. The

uptake of metformin in liver is dependent on organic cation transporter 1, and the glucose lowering effects seem to be affected by polymorphisms in the organic cation transporter 1 in some studies (Chen *et al.*, 2009).

Metformin also improves the insulin sensitivity of muscle tissue (Borst *et al.*, 2001), although the intestine has been considered to be a more important target of metformin action (Bailey *et al.*, 2008). Putative evidence speaks against the liver as the main target organ of metformin, which has raised some controversy. Metformin increases the concentrations of intestinal hormone glucagon-like peptide 1 (Mannucci *et al.*, 2001) and using a delayed-release formulation of metformin leads only to minimal plasma concentrations without curtailing the effect on fasting plasma glucose or HbA1c (Buse *et al.*, 2016). Also in contrast to previous studies, a large meta-analysis found that metformin transporter polymorphisms do not significantly affect metformin action (Dujic *et al.*, 2017). In a recent randomized trial, metformin increased the rate of glucose disposal and endogenous glucose production similarly in subjects with newly diagnosed T2DM and non-diabetic controls (Gormsen *et al.*, 2019). This effect might be due to non-oxidative glucose disposal following increased glucose uptake in the intestine. In the light of recent findings also gut microbiota may be involved in metformin action (reviewed in (Vallianou *et al.*, 2019)).

Clearly, there is controversy regarding the effects of metformin and to make matters even more complicated, the effects may differ depending on study subjects and duration of metformin treatment.

2.2.1 Metformin treatment in pregnancy

At first, metformin treatment during pregnancy was quite minimal and only a few studies were made, mostly in South Africa (Coetzee *et al.*, 1979, 1985). Still at the beginning of the 21st century conducting a controlled trial involving metformin in GDM was stated to be brave and even foolhardy (Dornan *et al.*, 2001). Nevertheless, after encouraging results from studies with patients with the polycystic ovary syndrome (PCOS) and T2DM were reported (Simmons *et al.*, 2004), a substantial controlled trial was made. A large multicenter randomized trial, the MiG trial, reported comparable perinatal outcomes in metformin and insulin-treated GDM (Rowan *et al.*, 2008). Similar results have since been obtained in other trials (Ijäs *et al.*, 2011; Spaulonci *et al.*, 2013; Tertti *et al.*, 2013; Zawiejska *et al.*, 2016).

Meta-analyses show that metformin treatment compared to insulin is not associated with short-term adverse outcomes and may even be beneficial in terms of lesser GWG and a lower risk of neonatal hypoglycemia, LGA babies and hypertensive disorders (Butalia *et al.*, 2017; Farrar *et al.*, 2017b). Although these results are reassuring, considerable differences between individual trials make it

difficult to draw firm conclusions. Respectively, besides different ethnic backgrounds of enrolled patients in the different trials, diagnostic and screening criteria, and treatment protocols of GDM have been heterogeneous. To resolve these uncertainties an individual patient data meta-analysis is planned (Mousa *et al.*, 2020).

The use of metformin has also been studied to prevent excessive fetal growth in obese women without GDM in the EMPOWaR and MOP trials (Chiswick *et al.*, 2015; Syngelaki *et al.*, 2016). Neither of the two trials demonstrated an effect on fetal growth, but GWG and the incidence of preeclampsia were lower in the metformin group compared to the group on placebo in the MOP trial (Syngelaki *et al.*, 2016). Why this effect was not seen in the EMPOWaR trial might be explained by a higher BMI threshold for trial entry ($\geq$ 35 vs. 30 kg/m^2) and by better adherence to treatment in the MOP trial.

Metformin has been studied in pregnancies complicated by T2DM, T1DM and PCOS. In patients with PCOS metformin may be used to treat infertility (Heard *et al.*, 2002) and retrospective data implies that continuation of metformin in these pregnancies after the first trimester may have some benefits (De Leo *et al.*, 2011). Metformin has, however, been deemed ineffective in preventing GDM in high risk subjects (Doi *et al.*, 2020). As in GDM, metformin may be used in T2DM (Butalia *et al.*, 2017; Feig *et al.*, 2020) and continuation of ongoing metformin treatment after conception in T2DM is endorsed by the Finnish diabetes association (Diabetesliitto) in their recommendation ”Diabeetikon hoito raskauden aikana” (Treatment of a diabetic during pregnancy) (Vääräsmäki *et al.*, 2012). Metformin is also studied in pregnancies with T1DM to examine if metformin ameliorates insulin resistance, but thus far data is very limited (Ping *et al.*, 2019).

Metformin crosses the placenta, and the drug concentrations are similar in the maternal and fetal circulation (Vanky *et al.*, 2005; Tertti *et al.*, 2010). Based on a large database and prospective data, metformin exposure during the first trimester does not seem to cause spontaneous abortions, or birth defects (Given *et al.*, 2018; Scherneck *et al.*, 2018).

In follow-up studies, metformin exposure has been related to increased offspring weight and BMI (van Weelden *et al.*, 2018; Hanem *et al.*, 2019), which has caused some concerns over the safety of metformin during pregnancy. Although the results between different follow-up studies have been similar, loss of offspring during follow-up may have introduced selection bias. Contrary to these findings, there were no differences in offspring weight at 4 years between metformin and insulin-treated pregnancies in a population cohort study from New Zealand (Landi *et al.*, 2019).

2.2.2 Effects of metformin on the serum metabolome

Early studies indicated that metformin may increase blood lactate, pyruvate, alanine and 3-hydroxybutyrate (Nattrass *et al.*, 1977, 1979), i.e., precursors of gluconeogenesis. The results were, however, inconsistent and the studies small (Nattrass *et al.*, 1977, 1979; Campbell *et al.*, 1987).

More recent studies have since shown that metformin treatment affects serum amino acids. Decreased concentrations of phenylalanine, tyrosine and valine (Huo *et al.*, 2009; Irving *et al.*, 2015; Preiss *et al.*, 2016; Rotroff *et al.*, 2016; Safai *et al.*, 2018) and increased alanine, isoleucine and leucine (Preiss *et al.*, 2016; Eppinga *et al.*, 2017; Safai *et al.*, 2018) have been reported. Walford *et al.* demonstrated that the effect of metformin on circulating amino acid concentrations is modified by the degree of insulin resistance of the study subjects (Walford *et al.*, 2013), which may explain some of the heterogeneity in the results.

Metformin decreases LDL cholesterol (Wulffelé *et al.*, 2004) and this effect may be caused by a decrease in acyl-alkyl phosphatidylcholines (36:4, 38:5 and 38:6) (Xu *et al.*, 2015; Breier *et al.*, 2017). Also, a more detailed lipid profile analysis showed that metformin treatment is associated with a decrease in the concentrations of several lysophosphatidylcholines (Cai *et al.*, 2009; Huo *et al.*, 2009) and sphingomyelins (Zhang *et al.*, 2014) and an increase in lysophosphatidylethanolamines (Safai *et al.*, 2018) and phosphatidylcholines (Zhang *et al.*, 2014).

The effects of metformin treatment on metabolism during pregnancy has been studied far less and metabolomic analyses have not been previously published. In the EMPOWaR study, obese pregnant women without diabetes were randomized to receive metformin or placebo at 12–16 gw. There were no differences in maternal NEFA, TG, HDL cholesterol and LDL cholesterol at 36 gw or in BW between the groups (Chiswick *et al.*, 2015). Additionally, maternal lipids have been evaluated in at least two randomized trials where metformin and insulin treatments in GDM are compared. In the MiG trial, maternal total, HDL and LDL cholesterols were not different between the treatment groups, but TG was considerably more elevated among the patients on metformin than on insulin (Barrett *et al.*, 2013a). Another analysis of the MiG population showed that maternal TG concentrations at 36 gw were related to HbA1c in the insulin group and to maternal ethnicity in the metformin group (Barrett *et al.*, 2013b).

In a smaller randomized trial from Poland, maternal TG was marginally higher in the metformin group than the insulin group, but this difference, as the differences in total cholesterol and HDL cholesterol, were not statistically significant between the groups (Zawiejska *et al.*, 2016).

Treatment of GDM with metformin does not seem to affect TG, HDL cholesterol or LDL cholesterol in cord blood (Barrett *et al.*, 2013a).

To summarize, metformin causes alterations in serum gluconeogenic substrates, amino acids, including alanine, isoleucine, leucine, phenylalanine, tyrosine and valine, LDL cholesterol and certain phospholipids. The data regarding effects of metformin on metabolome in pregnancy is currently mostly lacking.

2.2.3 Anti-inflammatory effects of metformin

Besides lowering blood glucose and altering amino acid and lipid metabolism, metformin seems also to suppress low-grade inflammation (Saisho, 2015). Metformin reduces the synthesis of proinflammatory cytokines, such as IL-6 and TNF-α, via NFκB inhibition (Saisho, 2015). These effects may be mediated both by AMPK-dependent and by AMPK-independent mechanisms (Saisho, 2015). GDM, as several other pathological conditions, is associated with low-grade inflammation. In clinical studies, metformin has been variably effective in ameliorating this inflammation.

In the Diabetes Prevention Program study, long-term treatment with metformin in patients at high risk of T2DM decreased CRP more than placebo (Goldberg *et al.*, 2014). Similarly, metformin treatment in PCOS was associated with a decrease in CRP (Wang *et al.*, 2017a). In GDM, however, metformin did not significantly affect maternal CRP as demonstrated in the MiG trial (Barrett *et al.*, 2013a).

Metformin suppresses IL-6 in vitro (Desai *et al.*, 2013; Han *et al.*, 2015), but not in vivo (in patients with PCOS) (Wang *et al.*, 2017a). In addition, metformin treatment reduced GlycA in a small group of patients with T2DM, but this was not the case in a larger study where the patients did not have diabetes (Eppinga *et al.*, 2017).

2.3 Metabolomics

2.3.1 Concept of metabolomics

Metabolomics refers to the study of metabolites. It comprises identification and quantification of small molecules (usually < 1.5 kDa) in a tissue, biological fluid, organ or a specific cell (reviewed in (Bain *et al.*, 2009) and (Lowe *et al.*, 2014)).

The metabolome, a metabolite profile of a given sample, can be either targeted or untargeted, depending on whether the intention is to analyze concentrations of a defined set of metabolites or the differences in an unknown number of analytes (Bain *et al.*, 2009). Either way, metabolomic analysis yields significant amounts of data to be analyzed. The benefits of the metabolomic approach are the possibility to discover novel biomarkers and to study changes in the metabolomic regulatory network, i.e., metabolic pathways (Bain *et al.*, 2009; Lowe *et al.*, 2014).

2.3.2 Common methods in metabolomic studies

The analysis techniques in metabolomics include NMR and mass spectrometry (MS), both of which have their own benefits (reviewed in (Zhang *et al.*, 2012) and (Dunn *et al.*, 2011). MS is often coupled with separation techniques, e.g., gas-chromatography and liquid chromatography (Zhang *et al.*, 2012).

NMR is a high-throughput quantitative method that can be largely automated and has good reproducibility (Soininen *et al.*, 2009). In addition, NMR can provide information regarding molecular structures (Zhang *et al.*, 2012). A drawback of NMR is its inability to detect very small quantities of a given metabolite.

MS, on the other hand, is more sensitive for low-concentration metabolites, but the analytical procedure is more complicated and prone to sources of error, and hence quality assurance throughout the analysis is important (Dunn *et al.*, 2011). MS requires control samples for qualitative analyses, which restricts the number of metabolites to be analyzed (Dunn *et al.*, 2011).

Metabolomic analysis yields significant amounts of data and with multiple single comparisons the risk of false positives, i.e., type I error increases. The goal is to identify the relevant information amid numerous associations. To overcome these hurdles, multivariate analysis methods, such as principal component analysis (PCA), partial least squares (PLS) regression or random forest analysis, may be applied (Dunn *et al.*, 2011). Another strategy is to adjust the p-values for multiple testing using, for example, the Bonferroni or Benjamini & Hochberg method. Importantly, the results should be validated, preferably in a separate dataset (Dunn *et al.*, 2011).

2.4 Maternal metabolome and fetal growth

2.4.1 Glucose metabolism

The hyperglycemia-hyperinsulinemia hypothesis, also known as the Pedersen hypothesis, is the most popular theory to explain the well-proven association between maternal hyperglycemia and BW (Catalano *et al.*, 2011). According to the hypothesis maternal hyperglycemia leads to fetal hyperglycemia via increased placental transfer of glucose. This causes increased insulin secretion by the fetal pancreas and the resulting fetal hyperinsulinemia, together with a high glucose level, promotes excess fetal growth. Because maternal hyperglycemia stimulates fetal hyperinsulinemia and hence lower fetal serum glucose and presumably greater glucose concentration gradient over the placenta, the fetus “steals” glucose from the mother (Figure 3) (Desoye *et al.*, 2016). Therefore, maternal glucose early in pregnancy has relatively greater impact on fetal growth compared to later maternal hyperglycemia. In the HAPO study, higher maternal glucose was associated with an

elevated risk of LGA and higher cord serum C-peptide with similar associations in OGTT glucose values at fasting, 1 h and 2 h (HAPO Study Cooperative Research Group *et al.*, 2008). Accordingly, treatment of maternal hyperglycemia does reduce the incidence of macrosomia (Hartling *et al.*, 2013), but despite good glycemic control the rate of macrosomia remains higher in the diabetic pregnancies (Evers *et al.*, 2002).

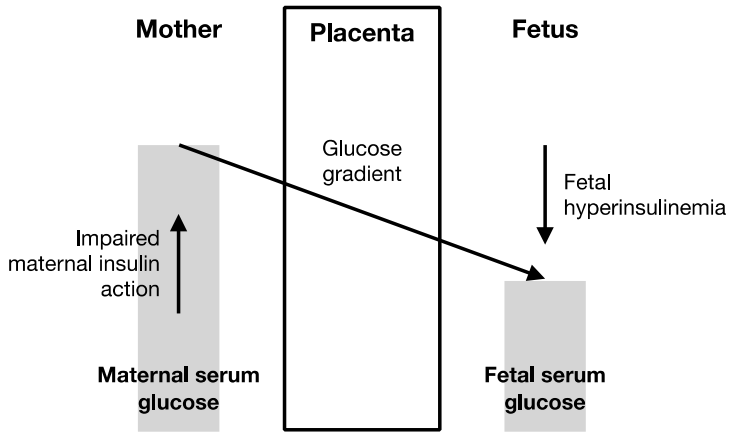

Figure 3. Elevated maternal glucose and fetal hyperinsulinemia both steepen the concentration gradient across the placenta thus facilitating increased glucose transfer. Modified from Desoye et al. (2016).

Obesity explains a proportion of LGA neonates in well-controlled GDM (Langer *et al.*, 2005b). Some of these differences between obese and normal weight subjects may, however, be related to glucose control. More novel glucose monitoring techniques, such as continuous glucose monitoring (CGM), enable more accurate detection of maternal hyperglycemia. In women without GDM, CGM showed that almost all glucose measures, except fasting glucose, are higher in obese subjects compared to normal weight subjects in late pregnancy (Harmon *et al.*, 2011).

To facilitate a meaningful interpretation of the CGM data, Law *et al.* applied functional data analysis to the glucose monitoring values and compared the differences in temporal glucose variation between normal pregnancies and pregnancies leading to delivery of a LGA infant (Law *et al.*, 2015). They found that in pregestational diabetes the glycemic features associated with LGA were different in each trimester. Using a similar approach in the third trimester in GDM they found that nocturnal hyperglycemia is associated with LGA (Law *et al.*, 2019). Use of CGM improves perinatal outcomes, reduces the incidence of LGA and leads to overall lower BW in GDM (Yu *et al.*, 2014).

Increasing insulin resistance in pregnancy decreases glucose utilization, increases lipolysis and leads to increased ketone concentrations. 3-hydroxybutyrate is the most abundant ketone body in the human blood circulation and during pregnancy it is further elevated by GDM (Montelongo *et al.*, 1992) and high maternal BMI (Hellmuth *et al.*, 2017a). 3-hyxroybutyrate crosses the placenta and has been suggested to promote fetal growth, but there is insufficient evidence to back-up this claim. Increased 3-hydroxybutyrate has, nevertheless, been positively associated with BW (Kadakia *et al.*, 2019a). In the large HAPO cohort, 3-hydroxybutyrate measured at 1 h after an OGTT glucose load, was positively related to BW and the sum of skinfolds of the neonate. These associations were attenuated when adjusted for maternal BMI or OGTT 1 h glucose (Kadakia *et al.*, 2019a).

In summary, maternal glucose, particularly in early pregnancy, is an important driver of fetal growth, and with novel methods such as CGM milder forms of hyperglycemia may be detected more accurately.

2.4.2 Amino acids

Amino acids are transferred across the placenta and, as glucose, are thought to promote fetal growth. In a study from the 1980's on a mixed population of healthy controls and subjects with T1DM plasma serine, threonine, lysine, proline, ornithine, arginine and total amino acids in late pregnancy correlated positively with BW (Kalkhoff *et al.*, 1988). Also, arginine was shown *in vitro* to stimulate fetal insulin secretion (Milner *et al.*, 1972).

More recently, two longitudinal cohorts of non-diabetic women showed that amino acid concentrations in the maternal circulation were not related to BW (Hellmuth *et al.*, 2017a, 2019). In the HAPO cohort, again, maternal serum amino acids in mid-pregnancy were predictive of BW (Kadakia *et al.*, 2019a). Thus, alanine, threonine, leucine/isoleucine, methionine, ornithine and proline measured 1 h after a glucose load in OGTT, but not at fasting, were positively related to BW (Kadakia *et al.*, 2019a). Proline was also related to the sum of skinfolds of the neonate. The association between leucine/isoleucine and BW was attenuated when adjusted for maternal BMI or OGTT at 1 h glucose (Kadakia *et al.*, 2019a).

Short-chain acylcarnitine metabolites of BCAA degradation are positively related to BW and cord blood C-peptide (Kadakia *et al.*, 2019a), although it is not known if these metabolites promote fetal growth directly or merely signal increased maternal insulin resistance.

In summary, increased concentration of maternal serum amino acids is associated with maternal insulin resistance, but their ability to directly stimulate fetal growth is uncertain.

2.4.3 Lipids

The importance of maternal lipids as fuel for fetal growth has been acknowledged for long (Freinkel, 1980), although the exact pathophysiology explaining the role of maternal lipids in excessive fetal growth is still not well understood (Herrera *et al.*, 2018).

Maternal NEFA (Sheath *et al.*, 1972) and TG are positively related to BW (Knopp *et al.*, 1992; Di Cianni *et al.*, 2005), but these associations may differ by patient characteristics. Schaefer-Graf *et al.* demonstrated a positive association between maternal late pregnancy NEFA and TG and neonate size in patients with GDM, but not in healthy controls (Schaefer-Graf *et al.*, 2008, 2011). Similarly, TG in late pregnancy was positively related to BW among overweight and obese subjects, but not among normal weight subjects (Misra *et al.*, 2011; Geraghty *et al.*, 2016). In the HAPO cohort, TG was associated with BW and the sum of neonate skinfolds (Kadakia *et al.*, 2019a). The association with BW was attenuated after adjustments for maternal BMI or glucose (Kadakia *et al.*, 2019a), as higher maternal BMI is associated with elevated TG (Sandler *et al.*, 2017). A Mendelian randomization analysis did not support a causal relationship between maternal TG and the BW of the neonates (Tyrrell *et al.*, 2016).

Two large cohorts show that maternal omega-3 and omega-6 FA in early and mid-pregnancy are related to fetal growth: the total phospholipid omega-3 to omega-6 FA ratio associated positively with fetal growth velocity and BW (Grootendorst-van Mil *et al.*, 2018), while maternal total omega-3 FA and dihomo-γ-linolenic acid (DGLA) were positively and arachidonic acid (ARA) inversely related to BW (Van Eijsden *et al.*, 2008; Grootendorst-van Mil *et al.*, 2018).

The association between the maternal lipidome and BW or neonate adiposity has been evaluated in three longitudinal studies (Hellmuth *et al.*, 2017b, 2019; LaBarre *et al.*, 2020). The number of lipid metabolites associated with BW increased as pregnancy proceeded in all studies. In the first trimester, NEFA were associated positively with BW and in late pregnancy phosphatidylcholines were inversely related to BW and neonatal adiposity (Hellmuth *et al.*, 2017b, 2019). Phosphatidylcholines, particularly those with an ether-bond or ARA, were associated with decreased neonatal adiposity (Hellmuth *et al.*, 2019). First-trimester NEFA were related to the phosphatidylcholine species that had a significant association with BW – this suggests that preconception dietary intake of fat by the mother may contribute significantly to fetal growth (Hellmuth *et al.*, 2019).

Maternal plasma HDL cholesterol correlated inversely with the BW in insulin-treated GDM patients (Barrett *et al.*, 2013a) and in overweight and obese subjects (Misra *et al.*, 2011). The detailed effects of maternal lipoprotein subfractions on fetal weight are poorly known.

In short, maternal serum lipids, TG and FA in particular, are related to fetal growth but this association seems to be affected by maternal obesity, GDM and gestational age at sampling.

2.4.4 Insulin-like growth factor-binding protein 1

Insulin-like growth factor-binding protein 1 (IGFBP-1) is one of the six binding proteins regulating the bioavailability of insulin-like growth factor 1 (IGF-1) – a potent growth promoting hormone (Baxter, 1995) and it is expressed in liver and placenta (The Human Protein Atlas; Uhlén *et al.*, 2015). While most of the circulating IGF-1 binds to IGFBP-3, the concentration of IGFBP-1 changes in response to glucose and insulin (Baxter, 1995). Thus, IGFBP-1 is thought to participate in the regulation of glucose metabolism. In the non-pregnant population a low concentration of IGFBP-1 is related to the metabolic syndrome (Heald *et al.*, 2003), risk of T2DM (Lewitt *et al.*, 2010), hepatic fat content and hepatic insulin resistance (Kotronen *et al.*, 2008).

Phosphorylation of IGFBP-1 increases its affinity to IGF-1 and the highly phosphorylated IGFBP-1 isoform (high-pIGFBP-1) prevails normally. During pregnancy, however, also less phosphorylated IGFBP-1 (low-pIGFBP-1) is detected (Westwood *et al.*, 1994). The fasting plasma IGFBP-1 concentrations are elevated in early pregnancy and remain elevated until postpartum (Clapp *et al.*, 2004; Larsson *et al.*, 2013).

Based on studies in pregnancy, IGFBP-1 is inversely related to the subject's body fat percentage, body weight (Olausson *et al.*, 2010) and insulin resistance (Ramirez *et al.*, 2014) and is lower in subjects with incipient GDM (Qiu *et al.*, 2005) and GDM (Ramirez *et al.*, 2014; Lappas, 2015). IGFBP-1 has also been inversely related to BW in some (Jansson *et al.*, 2008; Åsvold *et al.*, 2011; Lappas, 2015), but not in all studies (Clapp *et al.*, 2004).

2.5 Maternal metabolome and adverse pregnancy outcomes

There is evidence to support a causal relationship between certain features in the maternal metabolome and fetal growth (Freinkel, 1980; Catalano *et al.*, 2011) and, as reviewed above, maternal GDM is associated with distinct alterations in the metabolome, which are likely to be due to underlying obesity and insulin resistance. Besides fetal growth, maternal metabolomics can be applied to study the origins of and predict adverse outcomes, such as fetal growth restriction (FGR) and preeclampsia (Fanos *et al.*, 2013; Dessì *et al.*, 2015; Leite *et al.*, 2019). The associations between the maternal metabolome and fetal anomalies (Diaz *et al.*,

2011) and preterm birth (Carter *et al.*, 2019) have also been studied. The methodology between different studies varies considerably, thus precluding the identification of universally adverse metabolic features.

2.5.1 Fetal growth restriction

FGR is a disorder of fetal growth and notwithstanding the heterogenous etiologies (American College of Obstetricians and Gynecologists, 2013), metabolic traits associated with FGR are related to lipid and energy metabolism (Leite *et al.*, 2019), similarly as the associations between maternal obesity, GDM and fetal growth. However, the heterogeneity of study populations and study methods precluded the possibility to conduct a meta-analysis in a recent systematic review (Leite *et al.*, 2019). In a large longitudinal study, four metabolites (1-(1-enyl-stearoyl)-2-oleoyl-GPC (P-18:0/18:1); 1,5-anhydroglucitol; 5α-androstan-3α,17α-diol disulfate and *N*1,*N*12-diacetylspermine) improved the detection rate of FGR (Sovio *et al.*, 2020a). The authors suggested that these unconventional biomarkers could reflect placental growth or trophoblast function.

2.5.2 Preeclampsia

In preeclampsia, increased concentrations of amino acids (arginine, methionine, alanine, phenylalanine and glutamate) have been reported in maternal serum or plasma (Benny *et al.*, 2020). Alterations of lipid metabolism have also been reported in small studies involving elevated lysophosphatidylcholines and phytosphingosine and decreased concentrations of the D-vitamin metabolite 1,25-dihydroxyvitamin D3-26,23-lactone and of omega-3 FA derived eicosanoids (Liu *et al.*, 2019; Tan *et al.*, 2020). Sander *et al.* used less common metabolites detected by untargeted metabolomics approach and demonstrated a good separation of third trimester plasma samples between patients with preeclampsia and controls (Sander *et al.*, 2019).

Most of the studies were rather small and a separate validation dataset was not used. In a large study, Kenny *et al.* found that in early pregnancy maternal plasma the concentrations of several carnitines, FA and phospholipids were elevated in the preeclampsia group and, using a separate validation cohort, they found that a combination of 14 metabolites was able to predict preeclampsia (area under receiver operator characteristic curve = 0.92) (Kenny *et al.*, 2010). In another study, a validation cohort also resulted in the observation that mid-pregnancy phospholipids predict preeclampsia (Lee *et al.*, 2020). Based on a large longitudinal dataset and external validation, 4-hydroxyglutamate improved the prediction of preeclampsia at

term over the more commonly used ratio between soluble FMS-like tyrosine kinase 1 and placental growth factor (Sovio *et al.*, 2020b).

To conclude, preeclampsia is associated with changes in the maternal metabolome, but the heterogeneity of preeclampsia (Benton *et al.*, 2018) confounds the identification of robust biomarkers. However, given the potency of low-dose aspirin to prevent early preeclampsia and the novel screening algorithms for identification of patients sensitive to this treatment (Rolnik *et al.*, 2020), incorporating metabolomics data could further improve the efficacy and individualization of the treatment of preeclampsia.

2.5.3 Preterm birth

In a recent systematic review, preterm birth was inversely associated with myoinositol, creatinine, histidine and 5-oxoproline (Carter *et al.*, 2019). However, the studies were heterogeneous and amniotic fluid was used for sampling more often than maternal blood. In a study focusing on metabolomic markers of preterm birth in women with GDM or T2DM, several metabolites were significantly associated with gestational age at delivery after adjustment for false discovery rate (FDR) (Diboun *et al.*, 2020). TG and diacylglycerols containing oleic acid and LA were the metabolites that were most strongly inversely related to the length of gestation at delivery. In this analysis the authors did not find significant associations between maternal metabolites and other perinatal outcomes, including preeclampsia, intrauterine fetal demise, and macrosomia (Diboun *et al.*, 2020).

2.6 Cord blood metabolome

2.6.1 Placental function and transfer of nutrients

The fully developed placenta consists of a chorionic plate on the fetal and a basal plate on the maternal side. In between are the placental villi that contain the fetal capillary circulation. The placental villi are surrounded by an intervillous space, which is filled with maternal blood supplied by maternal spiral arteries. The exchange between respiratory gases and nutrients occurs in these placental villi and, to facilitate effective transport, there are only two layers of cells dividing maternal and fetal circulations: fetal capillary endothelial cells and syncytiotrophoplasts. On the fetal side, two umbilical arteries bring blood to the placenta and the oxygenated blood leaves the placenta through a single umbilical vein to the fetus.

While respiratory gases O_2 and CO_2 are transported via diffusion, there are several transportation mechanisms for nutrients and macromolecules in the placental villi (Burton *et al.*, 2016). Sampling of umbilical blood is achieved by collecting

either venous, arterial, or mixed blood. Arterial blood is representative of fetal metabolism, while placental transportation of nutrients is reflected in venous blood. In a mixed blood sample, the proportions of arterial and venous blood may vary to some degree, but the differences in amino acid, fatty acid and cholesterol concentrations between umbilical arterial and venous samples are usually minimal (Cetin *et al.*, 2005; Ortega-Senovilla *et al.*, 2009; Horne *et al.*, 2019).

GLUT1 is the main glucose transporter in the human placenta (Jansson *et al.*, 1993). Its expression in the syncytiotrophoplast basal membrane is increased in pre-gestational diabetes (Gaither *et al.*, 1999; Jansson *et al.*, 1999), but probably not in GDM (Jansson *et al.*, 2001; Stanirowski *et al.*, 2017). Increased expression of GLUT4 and GLUT9 transporters has been established in patients with GDM and pre-gestational diabetes alike (Stanirowski *et al.*, 2017), but the significance of glucose transporters other than GLUT1 is unclear.

Amino acids are transported across the placenta by a variety of transporter proteins (Jansson, 2001). Systems A and L are the most studied in the placenta, although it is not clear whether system A or L is altered in GDM or pre-gestational diabetes (Kuruvilla *et al.*, 1994; Jansson *et al.*, 2002; Nandakumaran *et al.*, 2004). Nevertheless, amino acid transport seems to be altered in GDM (Cetin *et al.*, 2005) and modulated by insulin and leptin (Jansson *et al.*, 2003; Ericsson *et al.*, 2005) and inflammatory cytokines IL-6 and TNF-α (Jones *et al.*, 2009).

Most of the lipids in the maternal circulation are bound to lipoprotein particles and, in order to cross the placenta, placental trophoblasts need first to bind the lipoproteins. Thereafter TG and phospholipids are hydrolyzed by placental lipases to release NEFA. Correspondingly, receptors for HDL, LDL (Cummings *et al.*, 1982) and VLDL (Wittmaack *et al.*, 1995) are expressed in the placenta. Several lipases, including endothelial lipase, LPL, hormone sensitive lipase and adipose triglyceride lipase, have been detected in the placenta (Gauster *et al.*, 2007; Barrett *et al.*, 2014). In the term placenta, LPL activity is increased in pregnancies complicated by GDM and maternal obesity (Dubé *et al.*, 2012, 2013). Endothelial lipase expression may be increased in GDM (Radaelli *et al.*, 2009; Gauster *et al.*, 2011), although not according to all studies (Barrett *et al.*, 2014; Ruiz-Palacios *et al.*, 2017). Inside the trophoblasts, NEFA are carried by fatty acid binding proteins, the regulation of which is also altered in GDM (Magnusson *et al.*, 2004; Radaelli *et al.*, 2009).

2.6.2 Effects of gestational diabetes on the cord blood metabolome

A few trials have compared amino acids in the cord blood in GDM and normal pregnancies (Cetin *et al.*, 2005; Dani *et al.*, 2014; Shokry *et al.*, 2019b) and the

concentrations of alanine, arginine, asparagine, glutamic acid, histidine, lysine, methionine, ornithine, phenylalanine and valine were higher in the infants of mothers with GDM (Cetin *et al.*, 2005; Dani *et al.*, 2014). Cetin *et al.* found also that isoleucine and leucine were higher in the cord plasma of the GDM group (Cetin *et al.*, 2005). In accordance, HAPO data showed that cord plasma leucine/isoleucine is inversely related to maternal insulin sensitivity (Lowe *et al.*, 2017). However, in a recent study only isoleucine and leucine in cord plasma were *lower* in the GDM group (Shokry *et al.*, 2019b), although these associations were adjusted for confounding factors such as GWG (Shokry *et al.*, 2019b), unlike previous comparisons (Cetin *et al.*, 2005; Dani *et al.*, 2014). Still, it seems unlikely that this could have such a drastic effect on the results.

In umbilical venous plasma, the percentages of individual FA out of total FA did not differ between GDM and non-GDM, but ARA, total omega-6 PUFA, DHA, total omega-3 PUFA and total PUFA were lower in the umbilical arterial plasma in the GDM group (Ortega-Senovilla *et al.*, 2009). In a larger cohort, umbilical cord arterial serum FA concentrations were lower in the GDM group, with the exception of ALA which was higher in the GDM than in the non-GDM group (Ortega-Senovilla *et al.*, 2020). The feto-maternal ratio of γ-linolenic acid (GLA) and ALA were higher and DGLA, ARA and DHA lower in the GDM group (Ortega-Senovilla *et al.*, 2020). Whether these differences result from changes in placental transfer and metabolism or from fetal handling of FA is speculative.

Cord blood total NEFA are increased in maternal GDM, but TG, total cholesterol, HDL and LDL cholesterol are not affected (Schaefer-Graf *et al.*, 2011; Houde *et al.*, 2014).

Using NMR analysis, Dani *et al.* found umbilical cord arterial serum glucose to be lower and pyruvate, α-ketoisovaleric acid, hypoxanthine and overall lipid and lipoprotein content to be higher in the neonates of the GDM group (Dani *et al.*, 2014). In another prospective cohort study, NEFA C26:1, acyl-alkyl-phosphatidylcholine C38:0 and carnitine were lower in cord venous plasma in the GDM compared to non-GDM group (Shokry *et al.*, 2019b). The authors suggested that FA oxidation was reduced in the neonates exposed to GDM. However, in a large HAPO cohort, the levels of acylcarnitines in the cord plasma were inversely associated with maternal insulin sensitivity and positively associated with maternal OGTT 1 h glucose (Lowe *et al.*, 2017). These findings suggest increased rather than decreased BCAA and FA metabolism in neonates exposed to GDM.

2.6.3 Associations with birth weight

Schaefer-Graf *et al.* demonstrated that cord blood TG and NEFA are inversely related to BW in GDM, but in healthy controls the relation is positive (Schaefer-Graf

et al., 2008, 2011). An inverse association between cord blood TG and BW has also been found in studies of obese subjects (Patel *et al.*, 2018) and women who have given birth to a macrosomic infant (BW ≥ 4000g) (Geraghty *et al.*, 2016). Also analyses of the HAPO cohort found an inverse association between cord blood TG, but not NEFA (Kadakia *et al.*, 2019b). When using IADPSG criteria retrospectively, most of the included patients (1277 of 1600) in this subset of the HAPO cohort did not have GDM and the study was deemed underpowered to study these associations in the GDM group alone (Kadakia *et al.*, 2019b). The inverse association between TG and BW has been thought to be due to increased adipose tissue uptake of TG in large fetuses. Lower FA concentrations in the umbilical cord arterial serum in GDM compared to controls also fits with the hypothesis of enhanced fetal uptake of lipids (Ortega-Senovilla *et al.*, 2020). Cord blood TG is higher in SGA neonates compared to neonates with BW appropriate for gestational age (Wang *et al.*, 2007).

Of individual FA, omega-3 FA, DHA, omega-6 FA and total PUFA have been inversely and MUFA positively related to BW (Rump *et al.*, 2001; Hellmuth *et al.*, 2017c; Robinson *et al.*, 2018). Cord plasma ARA in TG and in cholesterol esters has been positively related to BW (Elias *et al.*, 2001), while the association between BW and cord plasma phospholipid ARA was either negative (Rump *et al.*, 2001) or absent (Elias *et al.*, 2001).

In three large metabolomic analyses, cord blood lysophosphatidylcholines have been associated positively and NEFA inversely with BW (Hellmuth *et al.*, 2017c; Lu *et al.*, 2018; Patel *et al.*, 2018; Robinson *et al.*, 2018). In a detailed analysis of cord vein lipidome lysophosphatidylcholines and lysophosphatidylethanolamines were positively related to BW, independent of FA chain length or the number of double bonds (LaBarre *et al.*, 2020). The mechanism for the association between lysophosphatidylcholines and BW remains unknown. In a cohort focusing on these associations separately in small groups of normal weight, overweight/obese and GDM mothers, lysophosphatidylcholines were positively related to BW in normal weight mothers and phosphatidylcholines were associated positively with BW in normal weight and inversely in overweight/obese and GDM mothers (Shokry *et al.*, 2019a).

An early study comparing SGA neonates and normally grown neonates at term reported decreased amino acid concentrations in umbilical venous and arterial plasma in the SGA group (Cetin *et al.*, 1988). These findings were not confirmed in a more recent study on SGA neonates (Miranda *et al.*, 2018).

In a prospective study of obese pregnant women, PCA projection of cord blood amino acids and related metabolites was not associated with BW (Patel *et al.*, 2018). Similarly, amino acids in two large cohorts were not associated with BW after neither Bonferroni nor FDR adjustment (Hellmuth *et al.*, 2017c; Lu *et al.*, 2018).

Prior to these statistical corrections histidine was positively and alanine inversely related to BW (Hellmuth *et al.*, 2017c).

In the HAPO cohort several amino acids, including arginine, glutamic acid, glycine, histidine, leucine/isoleucine, ornithine, proline, serine and threonine in cord blood were positively related to BW (Kadakia *et al.*, 2019b). These associations were, however, not significant when subjects retrospectively diagnosed with GDM were excluded. In a smaller cohort of normal uncomplicated pregnancies, cord blood amino acids were not related to cord blood leptin nor C-peptide nor neonatal adiposity (Kadakia *et al.*, 2018). In both studies medium and long-chain acylcarnitines were inversely related to the concentration of C-peptide in the umbilical cord (Kadakia *et al.*, 2018, 2019b).

Cord blood acylcarnitines, physiologically involved in FA β-oxidation and BCAA metabolism, have been positively associated with BW in some (Kadakia *et al.*, 2018, 2019b; Lu *et al.*, 2018) but not all studies (Patel *et al.*, 2018; Robinson *et al.*, 2018; Shokry *et al.*, 2019a). Perng *et al.* used an innovative approach to study the association between BW and BCAA metabolism (Perng *et al.*, 2017). They created a projection of the relevant metabolites, based on a PCA model of their previous study, in which BCAA metabolism was associated with childhood obesity. This PCA projection of BCAA metabolites associated with childhood obesity was associated with BW adjusted for gestational age (Perng *et al.*, 2017).

Cord blood 3-hydroxybutyrate and its metabolite, acylcarnitine C4-OH, were related to BW (Kadakia *et al.*, 2019b). Although fetal ketogenesis is minimal, the ketones in the fetal blood circulation could be used for fetal lipogenesis (Herrera *et al.*, 2006).

Also the intermediates of the tricarboxylic acid (TCA) cycle (Perng *et al.*, 2017), of purine/pyrimidine metabolism (Perng *et al.*, 2017; Kadakia *et al.*, 2019b) and of tryptophan metabolism (Perng *et al.*, 2017; Robinson *et al.*, 2018; Kadakia *et al.*, 2019b) in umbilical cord blood have been associated with BW.

In conclusion, fetal BW is reflected in the cord blood metabolome, and lipid as well as amino acid metabolism seems to be involved. These associations might not be the same in uncomplicated pregnancies, in SGA neonates and in pregnancies complicated by GDM.

2.6.4 Association with long-term outcomes

In the 1990's Hales and Barker presented the "thrifty phenotype" hypothesis (Hales *et al.*, 1992), according to which fetal undernutrition leads to adverse outcomes later in life (Barker *et al.*, 1993). It was proposed that the fetal adaptation to undernutrition could be disadvantageous later in postnatal life when nutrition is more likely to be abundant than restricted (Hales *et al.*, 2001). A few years later the concept of

developmental origins of health and disease or "DOHAD" was proposed (Gillman, 2005), focusing not only on exposure during the fetal stages of development, but also on perinatal and early life development. Besides growth restriction, fetal overnutrition, or LGA, is associated with adverse outcomes such as an increased risk of T2DM (Harder *et al.*, 2007). The mechanism by which prenatal and early life exposures affect future health are at least partly related to epigenetic programming (Słupecka-Ziemilska *et al.*, 2020).

The neonatal metabolome reflects fetal metabolism and could serve as an early marker of future morbidity. Indeed, several studies have found associations between cord blood metabolites, childhood obesity and diabetes risk.

Cord blood HDL, but not LDL or total cholesterol, was inversely related to infant weight at 6 months after adjustment for confounding factors (Geraghty *et al.*, 2016).

A Dutch birth cohort follow-up study examined the association between cord plasma phospholipid PUFA and insulin resistance when the children were 7 years old (Rump *et al.*, 2002). Low GLA and DGLA were associated with higher insulin concentration and insulin resistance and GLA with proinsulin and fat mass but not with BMI nor weight. The inverse association with GLA and fasting insulin was strongest in subjects with BW in the lowest tertile (Rump *et al.*, 2002). LA, ARA, eicosapentaenoic acid (EPA), DHA, total omega-6 FA or total omega-3 FA were not related to the outcome measures. Similarly, a pooled analysis of two cohorts failed to prove any consistent association between cord blood phospholipid PUFA and childhood obesity (Stratakis *et al.*, 2019).

Two follow-up studies of a German cohort have evaluated the association between cord serum long-chain PUFA in glycerophospholipids (Standl *et al.*, 2014), a targeted cord serum metabolome (Hellmuth *et al.*, 2017c) and childhood BMI. Based on this data the ratio of cord serum glycerophospholipid omega-6 to omega-3 was inversely associated with BMI when the children were 2 years and positively when they were 10 years of age (Standl *et al.*, 2014). In the other study, follow-up was continued for until 15 years. Alanine, histidine, several NEFA and omega-3 glycerophospholipid FA were predictive for postnatal weight gain (between 0 and 6 months) and alanine and NEFA were positively associated with BMI at age of 15 years. Yet, none of the associations between cord serum metabolites and postnatal weight measures in this study were statistically significant after Bonferroni correction (Hellmuth *et al.*, 2017c). On the contrary, Shokry *et al.* found various long-chain NEFA and phosphatidylcholines to be inversely related to post-natal growth, although this study lacked statistical power after it had divided the population into subgroups and multiple outcomes were examined (Shokry *et al.*, 2019a).

In a small case-control study, the associations between the umbilical cord plasma metabolome, accelerated postnatal weight gain and mid-childhood obesity were

evaluated (Isganaitis *et al.*, 2015). Although some differences were identified, they were not significant after FDR adjustment, and partial least squares discriminant analysis (PLS-DA) failed to provide sufficient separation between the groups. There were also considerable differences in maternal age, pre-pregnancy BMI (pBMI), paternal BMI and breastfeeding duration between the groups (Isganaitis *et al.*, 2015).

Another prospective cohort included infants of obese women. Phosphatidylcholines, lysophosphatidylcholines 16:1 and 18:1 in particular, were associated with infant weight at 6 months and increased postnatal weight gain (Patel *et al.*, 2018).

In addition to early obesity and insulin resistance, several cord blood phosphatidylcholines have been found to predict T1DM (La Torre *et al.*, 2013; Orešič *et al.*, 2013).

In summary, cord blood metabolomics holds a promise of predicting metabolic morbidity later in life. NEFA, phosphatidylcholines, omega-3 and omega-6 FA and certain amino acids may be suitable markers. These associations are, however, complicated by confounding factors, e.g., BW, maternal GDM and obesity and thus far the studies have lacked statistical power.

2.7 Knowledge gaps in the current literature

More accurate classification and risk assessment between heterogeneous population of GDM patients could yield in more personalized approach and more efficient use of limited health care resources. Clinical algorithms to predict the need of antihyperglycemic medication have been developed (Barnes *et al.*, 2016), but as metabolome reflects maternal insulin resistance (Sandler *et al.*, 2017) and is directly related to fetal growth (Freinkel, 1980; Scholtens *et al.*, 2016), the use of metabolomics might be advantageous in identifying patients at risk of requiring pharmacological treatment and/or adverse perinatal outcomes.

The current evidence claims metformin to be comparable, if not superior, to insulin regarding perinatal outcomes (Butalia *et al.*, 2017; Farrar *et al.*, 2017b). However, despite the decades of research, the effects of metformin are not fully elucidated. And in pregnancy there is paucity of data regarding the effects of metformin on maternal metabolome. In the MiG trial, metformin treatment compared to insulin treatment was shown to cause increased maternal plasma TG (Barrett *et al.*, 2013a). However, there is currently no data regarding the effects of metformin on maternal lactate, amino acids or lipids in more detailed lipoprotein subclasses. Moreover, while metformin did not affect serum CRP compared to insulin in GDM (Barrett *et al.*, 2013a), the number of patients was only 60–70 per group and data on other circulating markers of low-grade inflammation is completely lacking.

It is already known that maternal lipids are related to fetal growth (Freinkel, 1980; Herrera *et al.*, 2018) and this relationship seems to be dependent of maternal factors such as obesity (Misra *et al.*, 2011; Geraghty *et al.*, 2016) and GDM (Schaefer-Graf *et al.*, 2008, 2011). However, the studies of women with GDM have only or mostly included patients without pharmacological treatment (Knopp *et al.*, 1992; Schaefer-Graf *et al.*, 2008, 2011). And aside from the data derived from the MiG trial (Barrett *et al.*, 2013a), we do not know whether these associations between maternal lipids and BW are similar between the metformin and the insulin-treated patients. In theory, the beneficial effects of metformin in lowering the risk of macrosomia (Butalia *et al.*, 2017; Farrar *et al.*, 2017b; Feig *et al.*, 2020) could be mediated by changes in maternal metabolome.

Lastly, to date there is only one study that has compared neonate cord plasma TG, LDL cholesterol and HDL cholesterol between the metformin and the insulin-treated GDM patients (Barrett *et al.*, 2013a). Given that metformin has effects on metabolome (Cai *et al.*, 2009; Huo *et al.*, 2009; Zhang *et al.*, 2014; Irving *et al.*, 2015; Xu *et al.*, 2015; Preiss *et al.*, 2016; Rotroff *et al.*, 2016; Breier *et al.*, 2017; Eppinga *et al.*, 2017; Safai *et al.*, 2018) and it crosses the placenta (Vanky *et al.*, 2005; Tertti *et al.*, 2010), it would be important to characterize the potential metabolic alterations caused by the *in utero* exposure to metformin.

3 Aims

The principal aims of the study were to:

1. compare maternal metabolome, inflammatory markers and IGFBP-1 phosphoisoforms at the time of GDM diagnosis between patients who do or do not require pharmacological antihyperglycemic treatment, (Studies I–III)
2. examine the effects of metformin treatment, compared to insulin treatment, on the maternal serum metabolome and inflammatory markers in patients with GDM, (Studies I–III)
3. study the associations between the maternal serum metabolome, inflammatory markers and perinatal outcomes, especially birth weight, (Studies I–III) and
4. examine the effect of metformin exposure in utero on the umbilical cord serum metabolome and to evaluate the associations between the cord serum metabolome and birth weight. (Study IV)

4 Materials and Methods

4.1 Study population

Two hundred and seventeen women diagnosed with gestational diabetes were randomized to receive metformin (n = 110) or insulin (n = 107) in an open-label randomized trial (Tertti *et al.*, 2013). Additionally, women meeting the same inclusion and exclusion criteria but who did not require antihyperglycemic medication (n = 126) were included as a reference diet-only group. The study design and perinatal outcomes have been characterized previously in more detail in the studies by Tertti *et al.* (2013) and Pellonperä *et al.* (2016).

The study participants were recruited at Turku University Hospital (Finland) between June 2006 and December 2010. The diagnosis of GDM followed Finnish national criteria, and was based on a 2-hour 75 g oral glucose tolerance test (OGTT). The diagnostic cut-off values were ≥ 4.8 (fasting), ≥ 10.0 (1 h) and ≥ 8.7 mmol/l (2 h) until the release of new guidelines in December 2008, and thereafter ≥ 5.3, ≥ 10.0 and ≥ 8.6 mmol/l, respectively. At least two values above the cut-off values were required for diagnosis before and after the change in the cut-off values. The exclusion criteria included cardiac or renal insufficiency, liver disease, metformin use within 3 months preceding pregnancy or during pregnancy before the OGTT. The patients were diagnosed at mean 27 gw (SD = 2.6 weeks, range = 12–33 gw).

The trial was approved by the Ethics Committee of the Southwest Hospital District of Finland, the Finnish National Agency of Medicines and the European Union Drug Regulatory Agency (EUDRA). All study participants provided their informed consent and the trial was registered at ClinicalTrials.gov (NCT01240785, http://clinicaltrials.gov/ct2/show/NCT01240785). The original trial was powered to prove non-inferiority between the treatment groups in terms of BW, which was the primary outcome (Tertti *et al.*, 2013). The present study is a secondary analysis of the study population and an additional power-analysis was not deemed necessary.

The antihyperglycemic treatment was initiated if glycemic control was unsatisfactory despite diet and lifestyle modifications (two or more fasting plasma glucose values ≥ 5.5 mmol/l and/or postprandial values ≥ 7.8 mmol/l). Patients fulfilling these criteria were randomized by the physician using sealed envelopes.

Metformin was started with 500 mg once a day and increased up to 2000 mg per day if needed (median dose 1500 mg). Additional insulin was required by 23 patients in the metformin group to meet the glucose targets (fasting < 5.5 and 1 h postprandially < 7.8 mmol/l). In the analyses these patients are included in the metformin group unless otherwise specified. Insulin therapy was carried out with NPH insulin and/or rapid-acting insulin lispro or insulin aspart.

GWG was defined as the difference between the last measured maternal weight in the maternity welfare clinic and the self-reported weight before pregnancy. Late GWG was defined as weight gain from the initiation of antihyperglycemic treatment to the last measured weight. BW was measured in grams and converted into SD-units and percentiles (deviation from the mean value of the Finnish general population adjusted for gestational weeks (Pihkala *et al.*, 1989)). SGA and LGA were defined as BW below 10^{th} or above 90^{th} centile, respectively. Macrosomia was defined as BW ≥ 2.0 SD and/or ≥ 4500 g. Perinatal outcome data was collected from the medical records.

4.2 Analysis of serum samples

Baseline fasting venous serum samples were collected at study recruitment between 22 and 34 gw, at mean 30 gw, approximately two weeks after GDM had been diagnosed. An additional fasting serum sample was collected at 36 gw in the metformin and insulin groups, but not in the diet group. Umbilical cord serum samples were collected after delivery in all groups. C-peptide was determined only at baseline and HbA1c at baseline and at 36 gw (Table 4). Serum samples collected at baseline and at 36 gw and cord serum samples were stored at -70°C for further analyses of the metabolome, inflammatory markers and IGFBP-1 phosphoisoforms.

C-peptide and HbA1c values were determined from maternal serum samples using routine hospital laboratory methods

A targeted metabolome was measured using a high-throughput (^{1}H) NMR spectroscopy protocol (Soininen *et al.*, 2009). The NMR spectra of the cord serum samples were analyzed using a revised method validated also for cord serum samples (Würtz *et al.*, 2017). The targeted metabolome included amino acids, TG and cholesterol in lipoprotein subfractions, mean VLDL, LDL and LDL diameters, phosphoglycerides, cholines, sphingomyelins, apolipoproteins A-1 (apoA-1) and B (apoB), fatty acids and lactate. The cord serum metabolome was extended to include also ketones, detailed lipoprotein particle concentrations, total lipids and phospholipids in lipoproteins. These metabolites were selected based on the known association between these metabolites and the risk of T2DM and cardiovascular morbidity, as demonstrated earlier (Soininen *et al.*, 2015).

Analysis of GlycA was included in the targeted NMR metabolome. hsCRP and IL-6 were measured using enzyme-linked immunosorbent assay (ELISA) [human C-reactive protein (CRP) ELISA kit, R&D Systems, Minneapolis, USA; interleukin-6 (IL-6) ELISA kit, R&D Systems, Minneapolis, USA]. MMP-8, non-phosphorylated IGFBP-1 (non-pIGFBP-1), low-pIGFBP-1 and high-pIGFBP-1 were determined using ELISA and an immunoenzymometric assay, as described previously (Nuutila *et al.*, 1999; Kruit *et al.*, 2018).

Table 4. Serum sample analyses stratified by time point and treatment group.

	Baseline			**36 gestational weeks**			**Delivery**		
	Maternal serum			Maternal serum			Cord serum		
	Metf	Ins	Diet	Metf	Ins	Diet	Metf	Ins	Diet
C-peptide	X	X	X						
HbA1c	X	X	X	X	X				
NMR metabolome	X	X	X	X	X		X	X	X
Inflammatory markers	X	X	X	X	X				
IGFBP-1 phosphoisoforms	X	X	X	X	X				

Metf: metformin group, Ins: insulin group, Diet: diet group. NMR: nuclear magnetic resonance, IGFBP-1: insulin-like growth factor-binding protein 1

4.3 Statistical analyses

The R statistical software (versions 3.3.2 and 3.6.1, http://cran.r-project.org) was used for all statistical analyses and all figures were drawn using the *ggplot2* package in R. Comparisons of continuous clinical baseline and outcome data was carried out using either the t-test or the Mann-Whitney U test. For the comparison of categorical data the χ^2-test or Fisher's exact test was used. In the study comparing cord serum metabolomes (Study IV), all three (metformin, insulin and diet) groups were considered parallel and the continuous clinical data was thus compared by analysis of variance (ANOVA) and the Tukey's HSD test, or the Kruskall-Wallis test and the Dwass-Steele test. Otherwise the between group analyses were performed between the metformin and the insulin groups, or between the diet and the pharmacological treatment group (the pharmacological treatment group included pooled data from the metformin and insulin groups).

4.3.1 Study I

The metabolic variables, amino acids, glucose and lactate were compared between groups with the t-test or the Mann-Whitney U test and within group with the paired t-test or the paired Wilcoxon signed-rank test. The associations between amino acids, glucose, lactate and C-peptide, HbA1c and clinical outcome variables were studied using linear or logistic regression. Before regression analyses the continuous variables (except for BW which was already in SD-units) were centered and scaled. The regression models were adjusted for pBMI and smoking. The following outcomes were studied: GWG, hypertensive disorders (gestational hypertension or preeclampsia), gestational age at delivery, cesarean delivery, BW (adjusted for population mean for gestational age), SGA, LGA, NICU admission and need for intravenous (I.V.) glucose by the neonate. To control for type I error, FDR-adjustment (the Benjamini–Hochberg procedure) was applied to the regression analyses. A p-value below 0.05 was considered statistically significant.

4.3.2 Study II

The comparisons of IGFBP-1 phosphoisoforms and inflammatory markers between and within groups were performed as above. Correlations between IGFBP-1s, inflammatory markers, maternal age, pBMI and measures of glucose metabolism were examined using Spearman's correlation. The associations between IGFBP-1s, inflammatory markers and clinical outcome variables (total GWG, late GWG, hypertensive disorders, length of gestation at delivery, induction of labor, cesarean delivery, adjusted BW, SGA, LGA, NICU admission and neonatal I.V. glucose administration) were studied using linear or logistic regression. Before regression analyses the continuous variables (except for BW) were centered and scaled. The regression models were run unadjusted and adjusted for pBMI. Group-specific regression coefficients were provided, if pharmacological treatment interacted significantly ($p < 0.05$) with the association between the independent and the outcome variable in the regression model. The Bonferroni correction was applied on the regression analyses. A p-value below 0.05 was considered statistically significant.

4.3.3 Study III

Comparisons of maternal serum lipids between and within groups were made with the Mann-Whitney U and the Wilcoxon paired tests. The association between the maternal lipidome and BW was studied with both multivariate and univariate methods. Before these analyses the lipid values were centered and scaled and the missing values were imputed using the k-nearest neighbor method. A PLS regression

or PLS-DA was applied to study the association between the maternal lipidome and BW. PLS and PLS-DA analyses were completed using the *ropls* package for R (Thévenot *et al.*, 2015). The Q^2 value of the PLS / PLS-DA model was used to describe the amount of variation in the outcome variable that the model explains in internal cross-validation. In the univariate analyses the association between each lipid and BW was studied using linear or logistic regression. These regression analyses were also adjusted for pBMI, GWG and either baseline or 36 gw HbA1c. Separate regression coefficients for metformin and insulin groups were calculated, if there was significant ($p < 0.01$) interaction between the independent variable and the treatment. Due to the large number of comparisons in this set of intercorrelated data, $p < 0.01$ was considered statistically significant.

In a more detailed analysis birth weight centiles were compared between the treatment groups (metformin vs. insulin) in data stratified by maternal lipid quartiles using the Mann-Whitney U test. A comparison adjusted for confounding factors (pBMI, GWG and HbA1c) was performed using analysis of covariance (ANCOVA).

The associations between the maternal lipidome and other clinical outcome variables, GWG, length of gestation at delivery, hypertensive disorders (gestational hypertension or preeclampsia), cesarean delivery, NICU admission and neonatal I.V. glucose administration, respectively, were examined as described above using PLS and PLS-DA analysis and univariate regression analyses when appropriate.

4.3.4 Study IV

The metabolite concentrations in cord serum were compared between the metformin, insulin and diet groups using the Kruskall-Wallis and the Dwass-Steele test. Associations between metabolites and BW were studied with linear regression. The regression models were adjusted for mode of delivery, pBMI, GWG and maternal HbA1c at the time of GDM diagnosis. Regression coefficients were calculated separately for each treatment group, if there was a significant interaction term ($p < 0.05$) between the treatment group and the independent variable. Otherwise, due to the intercorrelated nature of the data and the amount of comparisons, a $p < 0.01$ was considered significant to decrease the risk of type I error.

5 Results

5.1 Population characteristics

There is slight variation in the number of individual metabolite assessments and between Studies I–IV due to logistics (insufficient amount of patient sample) and matters related to quality control. The missing measurements were similarly distributed across treatment groups and thus unlikely to cause bias in the results.

The descriptive statistics of the whole study population are provided in Table 5. The rate of induction of labor was higher in the insulin compared to the metformin ($p < 0.05$, Studies I–IV) or the diet group ($p < 0.001$, Study IV). In the diet group, the mean maternal age ($p < 0.05$, Study IV) and OGTT 1 h glucose ($p < 0.01$, Study IV) were lower compared to the metformin group and maternal HbA1c at baseline ($p < 0.05$, Study IV) and OGTT fasting glucose ($p < 0.05$, Study IV) values were lower in the diet group compared to the insulin group. When the diet group was compared to the combined pharmacological treatment group (Study I), maternal age ($p < 0.05$), HbA1c at baseline ($p < 0.01$), OGTT fasting glucose ($p < 0.01$), OGTT 1 h glucose ($p < 0.01$) and incidence of labor induction ($p < 0.01$) were lower in the diet group.

Table 5. Clinical characteristics of the study population. Modified from original publication I, Table 1, original publication II, Table 1 and original publication III, Table 1.

	Diet	**Insulin**	**Metformin**	**Drug groups combined**
n	*102–120*	*95–107*	*101–109*	*196–216*
Maternal characteristics				
Age (years)	30.3 ± 5.2	32.0 ± 5.5	31.9 ± 5.0	31.9 ± 5.2
Smoking	11 (9.3)	17 (16.0)	9 (8.6)	26 (12.3)
Primiparous	54 (45.0)	49 (45.8)	42 (38.5)	91 (42.1)
Pre-pregnancy BMI (kg/m^2)	29.0 ± 5.5	28.9 ± 4.7	29.5 ± 5.9	29.2 ± 5.3

(continued)

Table 5. Continued.

	Diet	Insulin	Metformin	Drug groups combined
Glucose metabolism				
HbA1c% at baseline	5.37 ± 0.32	5.51 ± 0.34	5.48 ± 0.34	5.49 ± 0.34
HbA1c at baseline (mmol/mol)	35.2 ± 3.5	36.7 ± 3.7	36.3 ± 3.7	36.5 ± 3.7
HbA1c% at 36 gw		5.69 ± 0.36	5.68 ± 0.33	5.68 ± 0.34
HbA1c at 36 gw (mmol/mol)		38.6 ± 3.9	38.5 ± 3.6	38.6 ± 3.7
OGTT fasting glucose (mmol/l)	5.38 ± 0.42	5.57 ± 0.42	5.52 ± 0.55	5.54 ± 0.49
OGTT 1 h glucose (mmol/l)	10.9 ± 1.0	11.2 ± 1.2	11.2 ± 1.5	11.2 ± 1.4
OGTT 2 h glucose (mmol/l)	7.86 ± 1.85	7.91 ± 1.75	8.33 ± 1.76	8.12 ± 1.77
Fasting C-peptide (nmol/l)	1.01 ± 0.31	1.05 ± 0.29	1.07 ± 0.33	1.06 ± 0.31
Pregnancy outcomes				
Gestational hypertension	4 (3.3)	4 (3.7)	2 (1.8)	6 (2.8)
Preeclampsia	4 (3.3)	10 (9.3)	5 (4.6)	15 (6.9)
Operative vaginal delivery	7 (5.8)	8 (7.5)	9 (8.3)	17 (7.9)
Cesarean delivery	19 (15.8)	18 (16.8)	15 (13.8)	33 (15.3)
Induction of labor	37 (30.8)	58 (54.2)	41 (37.6)	99 (45.8)
Total gestational weight gain (kg)	8.7 ± 5.2	7.8 ± 5.3	8.0 ± 5.2	7.9 ± 5.2
Late gestational weight gain (kg)		2.2 ± 3.0	1.8 ± 2.6	2.0 ± 2.8
Gestational age at delivery (weeks)	39.3 ± 2.2	39.4 ± 1.6	39.2 ± 1.4	39.3 ± 1.5
Neonatal outcomes				
Birth weight (g)	3550 ± 540	3590 ± 450	3610 ± 490	3600 ± 470
Birth weight (SD)	-0.045 ± 1.07	0.15 ± 0.96	0.17 ± 1.05	0.16 ± 1.00
Birth weight (centiles)	0.49 ± 0.29	0.54 ± 0.29	0.55 ± 0.29	0.55 ± 0.29
SGA	15 (12.5)	9 (8.4)	12 (11.4)	21 (9.9)
LGA	13 (10.8)	17 (15.9)	15 (14.3)	32 (15.1)
Macrosomia	5 (4.2)	1 (0.9)	5 (4.6)	6 (2.8)
Admission to NICU	34 (28.3)	39 (36.4)	33 (30.6)	72 (33.5)
Neonate I.V. glucose	27 (22.9)	25 (23.6)	25 (23.1)	50 (23.4)

Data is given as mean ± SD or n (%). SD: standard deviation, OGTT: oral glucose tolerance test, SGA: small for gestational age (birth weight < 10^{th} centile), LGA: large for gestational age (birth weight > 90^{th} centile), NICU: neonatal intensive care unit, I.V.: intravenous

5.2 Comparison of the maternal serum metabolome, inflammatory markers and IGFBP-1 phosphoisoforms between patients who required or did not require antihyperglycemic medication (Studies I–III)

Amino acids, glucose and lactate

At the time of GDM diagnosis, maternal serum glutamine was higher (390 vs. 370 μmol/l, $p = 0.009$) and glucose lower (3.9 vs. 4.1 mmol/l, $p < 0.0001$) in the diet group compared to the combined pharmacological treatment group. There were no differences in alanine, glycine, isoleucine, leucine, valine, phenylalanine, tyrosine, histidine or lactate.

Lipids

The median TG in small VLDL was marginally lower in the diet compared to the pharmacological treatment group (0.40 vs. 0.43 mmol/l, $p = 0.046$). Otherwise, maternal serum lipids at baseline did not differ between the patients in the diet and pharmacological treatment groups.

Inflammatory markers and IGFBP-1 phosphoisoforms

There were no differences in inflammatory markers or in IGFBP-1 phosphoisoforms at baseline between the patients who required pharmacological intervention and the patients who managed their glycemia with diet and lifestyle modifications only.

5.3 Maternal serum amino acid profile (Study I)

5.3.1 Associations between maternal serum amino acids, C-peptide and HbA1c

The associations were studied at the baseline for the whole study population (diet, metformin and insulin groups combined; n = 290–295 for C-peptide and 298–303 for HbA1c) and at 36 gw only in the pharmacological treatment group (n = 184–185 for HbA1c, C-peptide was not available at 36 gw).

At baseline, fasting C-peptide was significantly related to several amino acids: alanine (57; CI: 22, 92 pM C-peptide/SD alanine), isoleucine (72; CI: 41, 104 pM C-peptide/SD isoleucine), leucine (59; CI: 27, 93 pM C-peptide/SD leucine) and phenylalanine (76; CI: 44, 109 pM C-peptide/SD phenylalanine).

HbA1c was positively associated with phenylalanine (0.066; CI: 0.028, 0.10 HbA1c(%)/SD phenylalanine) at baseline. At 36 gw HbA1c was associated with alanine in metformin-treated (0.12; CI: 0.046, 0.21 HbA1c(%)/SD alanine) but not in insulin-treated patients (-0.020; CI: -0.097, 0.049 HbA1c(%)/SD alanine).

5.3.2 Effects of metformin versus insulin on maternal serum amino acids, glucose and lactate

At baseline, the metformin and insulin groups did not differ regarding their amino acid profiles. When the metformin and insulin groups were analyzed together, tyrosine did not change, glucose and valine decreased and lactate and the other amino acids increased from baseline to 36 gw (Table 6).

When the metformin and the insulin groups were analyzed separately, the decrease in valine was no longer significant (Table 6). The increases in alanine, glutamine, glycine, isoleucine, leucine and phenylalanine were significant in both groups, except for histidine which increased significantly only in the metformin group. Glucose declined and lactate rose similarly regardless of treatment group.

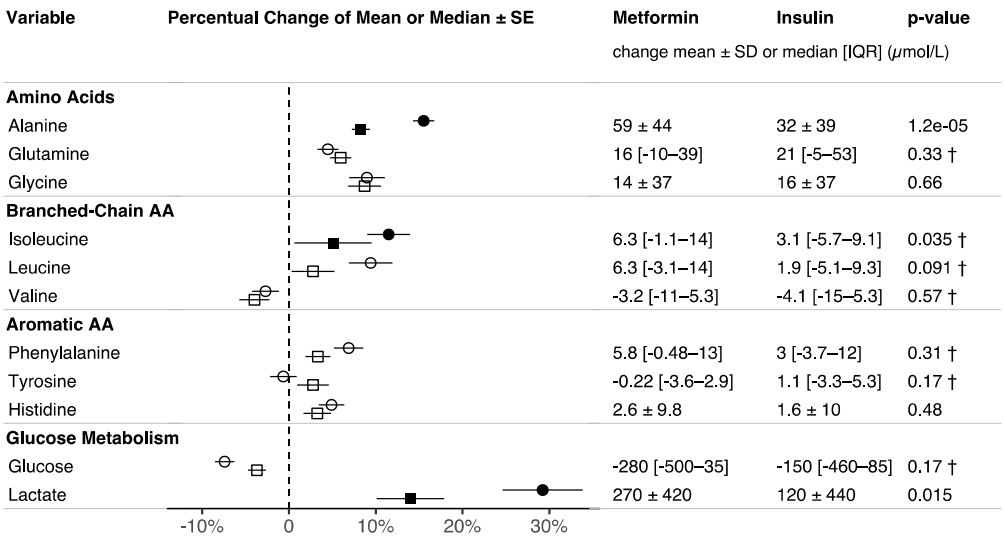

Figure 4. Comparison of changes in metabolic profiles from baseline to 36 gestational weeks between metformin and insulin treatment groups circles = metformin group, squares = insulin group, black square or circle denote significant p-value (< 0.05). p-value is given for the Mann-Whitney U (†) or the t-test where appropriate. Metformin n = 96–99, insulin n = 90–91. AA: amino acids, IQR: interquartile range, SE: standard error. Reproduced from original publication I, Figure 1.

Table 6. Maternal serum amino acid, glucose and lactate concentrations at baseline and at 36 gestational weeks by medication groups. Modified from original publication I, Table 3.

	Metformin and insulin combined			Metformin			Insulin		
	Baseline	**36 gw**	**p-value**	**Baseline**	**36 gw**	**p-value**	**Baseline**	**36 gw**	**p-value**
Amino acids									
Alanine	392 ± 44	438 ± 56	<0.0001	395 ± 45	454 ± 51	<0.0001	389 ± 44	421 ± 57	<0.0001
Glutamine	371 ± 56	384 ± 65	0.009	370 ± 55	381 ± 61	0.044	372 ± 58	387 ± 69	0.028
Glycine	207 ± 36	222 ± 40	<0.0001	201 ± 36	215 ± 36	<0.001	212 ± 36	229 ± 43	<0.0001
BCAA									
Isoleucine	53 [47–63]	59 [50–68]	<0.0001†	53 [47–61]	60 [48–70]	<0.0001†	56 ± 12	58 ± 13	0.041
Leucine	70 [64–79]	75 [68–84]	<0.0001†	72 ± 11	78 ± 14	0.0001	73 ± 12	76 ± 14	0.025
Valine	110 [97–124]	106 [96–120]	0.002†	112 ± 18	110 ± 19	0.19	109 ± 21	105 ± 21	0.056
Aromatic AA									
Phenylalanine	86 [78–94]	92 [85–97]	<0.0001†	86 ± 9.9	92 ± 9.6	<0.0001	87 [80–95]	92 [83–102]	0.002†
Tyrosine	37 [34–42]	39 [34–42]	0.48†	38 ± 6.6	38 ± 6.5	0.98	37 [34–42]	39 [35–44]	0.15†
Histidine	69 ± 9.6	71 ± 10	0.004	68 ± 8.8	71 ± 8.9	0.009	69 ± 10	71 ± 11	0.15
Glucose metabolism									
Glucose	4.1 ± 0.38	3.9 ± 0.43	<0.0001	4.1 ± 0.40	3.8 ± 0.43	<0.0001	4.1 ± 0.38	3.9 ± 0.43	0.003
Lactate	1.2 ± 0.31	1.4 ± 0.40	<0.0001	1.2 ± 0.34	1.5 ± 0.39	<0.0001	1.2 ± 0.28	1.4 ± 0.40	0.011

Metabolite concentrations are shown as mean ± SD or median [IQR] (μmol/l), glucose and lactate are mmol/l, p-value is given for Wilcoxon signed-rank (†) or t-test where appropriate, combined n = 186–190, metformin (including those who received metformin + insulin) n = 96–99, insulin only n = 90–91, AA: amino acids, BCAA: branched-chain amino acids, gw: gestational weeks, IQR: interquartile range. At baseline there were no significant differences between the two treatment groups (p > 0.05).

Comparing the changes between the metformin and the insulin groups showed that the alanine concentration rose considerably more in the metformin group (59 vs. 32 µmol/l, p<0.0001) (Figure 4). Isoleucine and lactate also rose more in the metformin group.

5.3.3 Associations between maternal serum amino acids, glucose and lactate at baseline and clinical outcome variables

Baseline analyses were performed for the whole study population. At baseline, besides glucose, isoleucine was positively related to BW (0.19; CI: 0.06, 0.31 SD/SD) (Table 7). Only glucose was associated with LGA (1.6; 1.2, 2.2 OR/SD) although this association was not significant after FDR-adjustment. Most of the amino acids were inversely related to the length of gestation at delivery (Table 7). After controlling for FDR, the associations were significant between alanine (-0.39; CI: -0.81, -0.18 weeks/SD), tyrosine (-0.39; CI: -0.82, -0.16 weeks/SD), glucose (-0.36; CI: -0.62; -0.15 weeks/SD) and length of gestation. Glycine and all three BCAA (isoleucine, leucine and valine) were inversely related to the length of gestation but none of these associations was significant after FDR-adjustment. Glutamine (-1.3; CI: -1.9, -0.64 kg/SD) was inversely and valine (0.58; CI: 0.049, 1.1 kg/SD) positively related to GWG, although the association between valine and GWG was not significant after the FDR-adjustment. Glucose was positively related to an increased risk of caesarean delivery. In the metformin group histidine was inversely related to GWG (-1.4; CI: -2.4, -0.38 kg/SD).

5.3.4 Associations between maternal serum amino acids, glucose and lactate at 36 gestational weeks and clinical outcome variables in patients requiring pharmacological treatment

At 36 gw, only alanine was associated with BW (0.15; CI: 0.029, 0.3 SD/SD) (Table 7) and this association was stronger in the metformin (0.31; CI: 0.11, 0.52 SD/SD) than the insulin (0.051; CI: -0.12, 0.28 SD/SD) group. Higher glucose was significantly associated with a lower incidence of SGA only before FDR-adjustment. Although not significant after adjusting for FDR, glutamine was positively associated with hypertensive disorders of pregnancy (2.3; CI: 1.3, 4.4 OR/SD).

Table 7. Summary of significant associations between amino acids, glucose, lactate and clinical outcomes 1. Modified from original publication I, Table 4.

Independent variable	Outcome	β-estimate [95% CI]	p-value	n
Baseline				
Glutamine	GWG (kg/SD)	-1.3 [-1.9; -0.64]	<0.0001 **	297
Valine	GWG (kg/SD)	0.58 [0.049; 1.1]	0.049	302
Alanine	Length of gestation (weeks/SD)	-0.39 [-0.81; -0.18]	<0.0001 **	303
Glycine	Length of gestation (weeks/SD)	-0.2 [-0.37; -0.049]	0.047	303
Isoleucine	Length of gestation (weeks/SD)	-0.23 [-0.44; -0.054]	0.023	303
Leucine	Length of gestation (weeks/SD)	-0.28 [-0.51; -0.086]	0.0054	303
Valine	Length of gestation (weeks/SD)	-0.22 [-0.42; -0.017]	0.027	303
Tyrosine	Length of gestation (weeks/SD)	-0.39 [-0.82; -0.16]	<0.0001 **	302
Glucose	Length of gestation (weeks/SD)	-0.36 [-0.62; -0.15]	0.00016 **	302
Glucose	Cesarean delivery (OR/SD)[a]	1.9 [1.3; 2.8]	0.00050 **	302 (43)
Isoleucine	Birth weight (SD/SD)	0.19 [0.06; 0.31]	0.0013 *	300
Glucose	Birth weight (SD/SD)	0.25 [0.12; 0.37]	<0.0001 **	299
Glucose	LGA (OR/SD)[b]	1.6 [1.2; 2.2]	0.0043	299 (44)
Glucose	NICU admission (OR/SD)[c]	1.4 [1.1; 1.8]	0.010	301 (95)
36 gestational weeks				
Glutamine	Hypertensive disorders (OR/SD)[d]	2.3 [1.3; 4.4]	0.0014	192 (20)
Alanine	Birth weight (SD/SD)	0.15 [0.029; 0.3]	0.047	190
Glucose	SGA (OR/SD)[e]	0.47 [0.25; 0.89]	0.026	190 (20)

Measures are expressed as odds ratios (OR) or regression β-estimates with 95% confidence intervals. At baseline, associations were estimated for the whole study population (diet and pharmacological treatment groups) and at 36 gw for the pharmacological treatment group. Adjustments were done for pre-pregnancy BMI and smoking. The reference groups for the binary outcomes were: a) vaginal delivery, b) birth weight < 90^{th} centile, c) no NICU admission, d) no hypertensive disorders (gestational hypertension or preeclampsia), e) birth weight > 10^{th} centile. * FDR adjusted $p < 0.05$, ** FDR adjusted $p < 0.01$. Number of subjects in each analysis (n) is expressed as total n, and n with a positive outcome in parentheses in case of binary outcome variables. GWG: gestational weight gain, SD: standard deviation, LGA: large for gestational age (adjusted birth weight > 90^{th} centile), SGA: small for gestational age (adjusted birth weight < 10^{th} centile), NICU: neonatal intensive care unit, OR: odds ratio, SD: standard deviation.

5.4 Maternal IGFBP-1 and inflammatory markers (Study II)

5.4.1 Correlations between inflammatory markers, age, BMI and measures of glucose metabolism in patients requiring pharmacological treatment

At baseline, pBMI correlated significantly and positively with hsCRP, IL-6 and MMP-8 as shown in Figure 5. Also C-peptide correlated significantly and positively with the inflammatory markers, hsCRP, IL-6 and GlycA. HbA1c correlated only with GlycA, and the OGTT glucose values or maternal age did not correlate with the inflammatory markers.

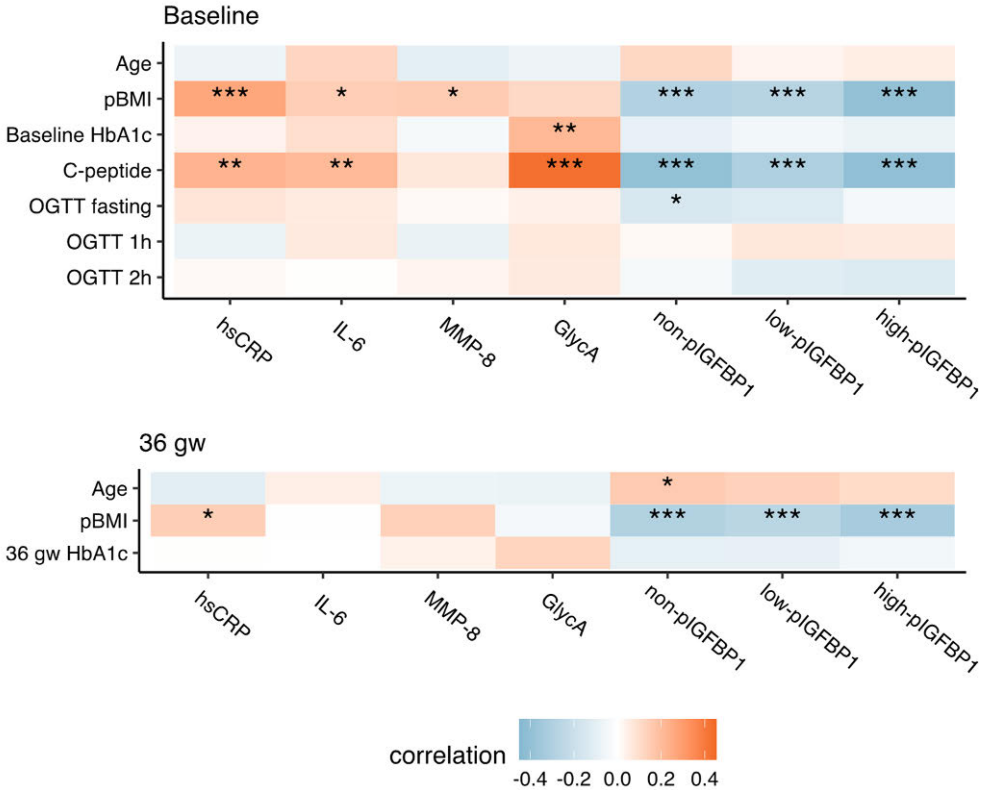

Figure 5. Heatmap representation of Spearman's correlations between age, pre-pregnancy BMI and glucose metabolism with inflammatory markers and IGFBP-1 phosphoisoforms at baseline (n = 196–208) and at 36 gestational weeks (n = 181–198). pBMI: pre-pregnancy body mass index, OGTT: oral glucose tolerance test, gw: gestational weeks, hsCRP: high sensitivity CRP, IL-6: interleukin 6, MMP-8: matrix metalloproteinase 8, GlycA: glycoprotein acetylation, non/low/high-pIGFBP-1: non/low/high-phosphorylated insulin-like growth factor-binding protein 1. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Reproduced from original publication II, Figure 1.

Both pBMI and C-peptide correlated strongly ($p<0.001$) and inversely with the IGFBP-1 phosphoisoforms (Figure 5). OGTT fasting glucose correlated inversely with non-pIGFBP-1.

At 36 gw the correlation between pBMI and hsCRP was attenuated but still significant ($p < 0.05$), and the inverse correlations between pBMI and non-pIGFBP-1 phosphoisoforms remained essentially unchanged compared to baseline (Figure 5). At 36 gw, but not at baseline, non-pIGFBP-1 correlated positively with maternal age.

Table 8. Change in concentrations of inflammatory markers and IGFBP-1 phosphoisoforms from baseline to 36 gestational weeks. Modified from original publication II, Table 2.

	Metformin and insulin combined		**Metformin**		**Insulin**		**p-value#**
n	*179*		*94*		*85*		
	median/mean [95% CI]	p-value	median/mean [95% CI]	p-value	median/mean [95% CI]	p-value	
Inflammation							
hsCRP (mg/l)	-0.47 [-1.3; -0.014]	0.011	-0.45 [-1.7; 0.16]	0.028	-0.47 [-1.8; 0.093]	0.18	0.72
IL-6 (ng/l)	0.70 [0.20; 1.40]	0.002	0.85 [0.50; 1.8]	0.002	0.62 [-0.19; 1.4]	0.13‡	0.31
MMP-8 (µg/l)	0.0 [-2.0; 0.80]	0.50	-0.70 [-2.0; 1.0]	0.76	0.70 [-2.0; 2.6]	0.20	0.28
GlycA (mmol/l)	0.11 [0.089; 0.13]	<0.0001	0.15 [0.11; 0.18]	<0.0001‡	0.091 [0.064; 0.12]	<0.0001‡	0.020
IGFBP-1							
non-phosphorylated (µg/l)	17.0 [13.0; 20.5]	<0.0001	21.0 [14.0; 26.0]	<0.0001	13.4 [7.9; 18.9]	<0.0001‡	0.008
low-phosphorylated (µg/l)	6.0 [4.0; 7.9]	<0.0001	6.0 [3.6; 7.5]	<0.0001	4.0 [-2.0; 4.0]	0.021	0.081
high-phosphorylated (µg/l)	300 [190; 410]	<0.0001‡	260 [110; 420]	0.001‡	340 [180; 500]	<0.0001‡	0.48†

Median/mean change from baseline to 36 gestational weeks [95% confidence interval (CI)]. Positive values denote increase and negative values decrease. p-values are given for the one-sample t-test (indicated with ‡) or Wilcoxon's signed rank test. For comparison of changes between metformin and insulin groups (#), Mann-Whitney U-test or the t-test (indicated with †) was used. hsCRP: high sensitivity CRP, IL-6: interleukin 6, MMP-8: matrix metalloproteinase 8, GlycA: glycoprotein acetylation, IGFBP-1: insulin-like growth factor-binding protein 1. n-values for GlycA are 190, 91 and 91 for combined, metformin and insulin groups, respectively.

5.4.2 Effect of metformin versus insulin on maternal serum inflammatory markers and IGFBP-1 phosphoisoforms

The concentration of low-pIGFBP-1 was lower at baseline in the metformin group than the insulin group (21.0 vs. 24.0 µg/l, p = 0.04). In the combined pharmacological treatment group, hsCRP decreased whereas IL-6 and GlycA increased from baseline to 36 gw (Table 8). When analyzed separately the decrease of hsCRP was significant only in the metformin group. Serum GlycA increased in both groups but significantly more in the metformin than the insulin group (0.15 vs. 0.091 mmol/l, p = 0.020). All three IGFBP-1 phosphoisoforms increased from baseline to 36 gw and this change was statistically significant in both groups. The increase of non-pIGFBP-1 was greater in the metformin group (21.0 vs. 13.4 µg/l, p = 0.008).

5.4.3 Associations between maternal serum inflammatory markers, IGFBP-1 and clinical outcome variables in patients requiring pharmacological treatment

The associations between inflammatory markers, IGFBP-1 phosphoisoforms, maternal outcomes (total GWG, late GWG, hypertensive disorders, induction of labor, caesarean delivery) and neonatal outcomes (BW, SGA, LGA, NICU admission, neonatal I.V. glucose treatment) were studied using regression analyses. A summary of the significant associations is given in Table 9. Notably, there were inverse associations between non-pIGFBP-1 and low-pIGFBP-1 and GWG. At baseline, non-pIGFBP-1 was inversely associated with total GWG both before (-1.2; CI: -2.0, -0.64 kg/SD) and after (-1.5; CI: -3.0, -1.2 kg/SD) adjustment for pBMI (Table 9) and low-pIGFBP-1 with total GWG only after adjustment for pBMI (-1.0; CI: -2.1, -0.64 kg/SD). At 36 gw, non-pIGFBP-1 was inversely related to total GWG in the unadjusted (-1.1; CI: -1.8, -0.52 kg/SD) and the adjusted regression model (-1.5; CI: -2.9, -1.3 kg/SD). Although the association was not significant after application of the Bonferroni-corrected threshold, baseline non-pIGFBP-1 was associated with BW in both unadjusted (-0.15; CI: -0.32, -0.052 SD/SD) and adjusted (-0.14; CI: -0.26, -0.0071 SD/SD) analyses. MMP-8 at baseline was associated positively with late GWG and at 36 gw inversely with BW. These association were not markedly affected by the adjustment, but were not significant after the Bonferroni correction.

Regression coefficients were calculated for the metformin and insulin groups separately if there was a significant interaction between the group and the independent variable ($p < 0.05$). None of these associations reached a Bonferroni-corrected p-value below 0.0045 (Table 10).

Table 9. Regression models with significant ($p < 0.05$) associations between inflammatory markers and IGFBP-1 concentrations, and maternal and neonatal outcomes. Modified from original publication II, Table 3 & Supplementary table 3.

Independent variable	Outcome	β-estimate [95% CI]	p-value	n
Unadjusted models				
Baseline				
non-pIGFBP-1	total GWG (kg/SD)	-1.2 [-2.0; -0.64]	<0.001*	201
MMP-8	late GWG (kg/SD)	0.41 [0.022; 0.77]	0.035	202
non-pIGFBP-1	late GWG (kg/SD)	0.45 [-0.87; -0.13]	0.021	202
hsCRP	length of gestation (weeks/SD)	0.2 [0.028; 0.36]	0.044	202
high-pIGFBP-1	induction of labor (OR/SD)[a]	0.67 [0.48; 0.92]	0.0094	202 (92)
non-pIGFBP-1	birth weight (SD/SD)	-0.15 [-0.32; -0.052]	0.027	198
36 gestational weeks				
Non-pIGFBP-1	total GWG (kg/SD)	-1.1 [-1.8; -0.52]	0.0027*	188
non-pIGFBP-1	late GWG (kg/SD)	-0.55 [-0.96; -0.21]	0.0069	189
non-pIGFBP-1	cesarean delivery (OR/SD)[b]	0.49 [0.24; 0.84]	0.043	189 (26)
MMP-8	birth weight (SD/SD)	-0.17 [-0.34; -0.037]	0.022	185
Models adjusted for pre-pregnancy BMI				
Baseline				
hsCRP	total GWG (kg/SD)	0.72 [0.55; 1.5]	0.0498	201
non-pIGFBP-1	total GWG (kg/SD)	-1.5 [-3.0; -1.2]	<0.0001*	201
low-pIGFBP-1	total GWG (kg/SD)	-1.0 [-2.1; -0.64]	0.0037*	201
MMP-8	late GWG (kg/SD)	0.43 [0.054; 0.80]	0.031	202
non-pIGFBP-1	late GWG (kg/SD)	-0.47 [-0.91; -0.17]	0.019	202
hsCRP	length of gestation (weeks/SD)	0.2 [0.035; 0.37]	0.048	202
non-pIGFBP-1	birth weight (SD/SD)	-0.14 [-0.26; -0.0071]	0.049	198
36 gestational weeks				
non-pIGFBP-1	total GWG (kg/SD)	-1.5 [-2.9; -1.3]	<0.0001*	188
low-pIGFBP-1	total GWG (kg/SD)	-0.99 [-2.1; -0.45]	0.0073	188
non-pIGFBP-1	late GWG (kg/SD)	-0.56 [-0.97; -0.22]	0.0075	189
MMP-8	birth weight (SD/SD)	-0.18 [-0.38; -0.063]	0.014	185

Both metformin and insulin-treated patients were included. Data is given as regression β-estimates or odds ratios (OR) in respect to one SD change of the predictor [95% confidence interval, CI]. The

reference groups in the binary outcomes were: a) no induction of labor, b) vaginal delivery. * p < 0.0045 (Bonferroni). The number of subjects in each analysis (n) is expressed as total n, and n with positive outcome in parentheses in case of a binary outcome variable. SD: standard deviation, gw: gestational weeks, GWG: gestational weight gain, pIGFBP-1: phosphorylated insulin-like growth factor-binding protein 1, MMP-8: matrix metalloproteinase 8, hsCRP: high sensitivity CRP.

Table 10. Regression models with significant (p < 0.05) interaction between treatment group (metformin or insulin) and the association between outcome and the independent variable. Modified from original publication II, Supplementary table 4.

Independent variable	Outcome	Model	Insulin	Metformin
Baseline				
low-pIGFBP-1	hypertensive disorders OR/SD	model 0	1.2 [0.58; 2.1] (0.56)	0.11 [0.01; 0.77] (0.053)
high-pIGFBP-1	hypertensive disorders OR/SD	model 0	1.2 [0.67; 2.2] (0.53)	0.28 [0.06; 0.56] (0.045)
low-pIGFBP-1	induction of labor OR/SD	model 0	0.97 [0.65; 1.4] (0.86)	0.38 [0.17; 0.74] (0.0066)
hsCRP	NICU admission OR/SD	model 0	0.59 [0.34; 1] (0.053)	1.3 [0.8; 1.9] (0.20)
hsCRP	NICU admission OR/SD	model 1	0.59 [0.33; 0.99] (0.052)	1.3 [0.76; 1.9] (0.24)
MMP-8	late GWG (kg/SD)	model 0	-0.24 [-0.82; 0.56] (0.35)	0.74 [0.18; 1.4] (0.035)
MMP-8	late GWG (kg/SD)	model 1	-0.24 [-0.84; 0.54] (0.35)	0.73 [0.16; 1.3] (0.039)
36 gestational weeks				
high-pIGFBP-1	hypertensive disorders OR/SD	model 0	1.5 [0.65; 3.3] (0.21)	0.41 [0.13; 1.2] (0.092)
hsCRP	length of gestation (weeks/SD)	model 0	-0.16 [-0.59; 0.024] (0.18)	0.4 [0.049; 0.68] (0.046)
hsCRP	length of gestation (weeks/SD)	model 1	-0.16 [-0.58; 0.027] (0.19)	0.41 [0.06; 0.69] (0.048)
non-pIGFBP-1	induction of labor OR/SD	model 1	1.1 [0.58; 1.7] (0.63)	0.49 [0.22; 0.88] (0.030)
high-pIGFBP-1	hypertensive disorders OR/SD	model 0	1.5 [0.65; 3.3] (0.21)	0.41 [0.13; 1.2] (0.092)

Data is given as regression β-estimates or odds ratios (OR) [95% confidence interval] (p-value). Model 0: unadjusted, model 1: adjusted for pre-pregnancy BMI. SD: standard deviation, NICU: neonatal intensive care unit, GWG: (maternal) gestational weight gain, pIGFBP-1: phosphorylated insulin-like growth factor-binding protein 1, MMP-8: matrix metalloproteinase 8, hsCRP: high sensitivity CRP. None of the p-values were below a Bonferroni-corrected threshold of 0.0045.

5.5 Maternal lipids (Study III)

5.5.1 Effect of metformin versus insulin on the maternal serum lipidome

Serum total TG and TG in VLDL, LDL and HDL particles increased from baseline to 36 gw in both treatment groups (Figure 6 and Figure 7). Except for the TG in HDL particles, the increases of TG were greater in the metformin group. Analysis of the detailed classification of the lipoprotein particles showed that TG rose significantly in all subclasses and in both treatment groups, with the exception of TG in large HDL particles in the insulin group. The increase was, again, greater in the metformin group and statistically significant in all HDL subclasses, small LDL and small to very large VLDL particles.

Serum total cholesterol rose in both treatment groups from baseline to 36 gw, but while the increase in TG was fairly uniform across lipoprotein subclasses, the change in cholesterol varied more by lipoprotein particle size. As TG, cholesterol increased in VLDL subclasses and this increase was significant in both treatment groups, with the exception of very small VLDL particles in the metformin group. The increase was greater in the metformin group than in the insulin group regarding medium to extremely large VLDL particles. Cholesterol concentrations in intermediate-density lipoprotein (IDL) and LDL particles remained relatively unchanged. While total HDL cholesterol in both groups decreased, this was mostly apparent in large and very large HDL particles. Medium HDL in the metformin group and small HDL in either treatment group did not change. When stratified into free and esterified cholesterols, both lipid classes increased similarly and independently of treatment, but the change was significant in both groups only for free cholesterol.

The mean VLDL particle diameter increased in both groups, but significantly more in the metformin-treated group. The average LDL particle diameter decreased only in the insulin group and the difference between the groups was significant. The mean HDL particle diameter decreased similarly in both groups.

All phospholipid species except sphingomyelins increased in both treatment groups. The increases in phosphoglycerides and the TG to phosphoglycerides ratio were greater in the metformin group.

The apoB concentration and apoB to apoA-1 ratio increased in both groups, while apoA-1 concentrations did not change from baseline to 36 gw.

Total FA increased in both treatment groups but more in the insulin group. PUFA increased significantly only in the metformin group whereas MUFA and SFA increased in both groups. The increases of MUFA and SFA were significantly greater in the metformin group. The proportion of PUFA (in relation to total FA) decreased and the proportions of MUFA and SFA increased in both groups. The decrease in

the proportion of PUFA and the increase in the proportions of MUFA and SFA were augmented in the metformin group. The estimated degree of unsaturation decreased in both groups.

The concentration of LA increased while the proportion of LA and DHA from total FA decreased in both groups. The concentrations of omega-6 FA increased but the proportion of both omega-3 and omega-6 FA decreased. There were no differences between the treatment groups in omega-3, LA and DHA. The proportion of omega-6 FA of total FA decreased more in the metformin group.

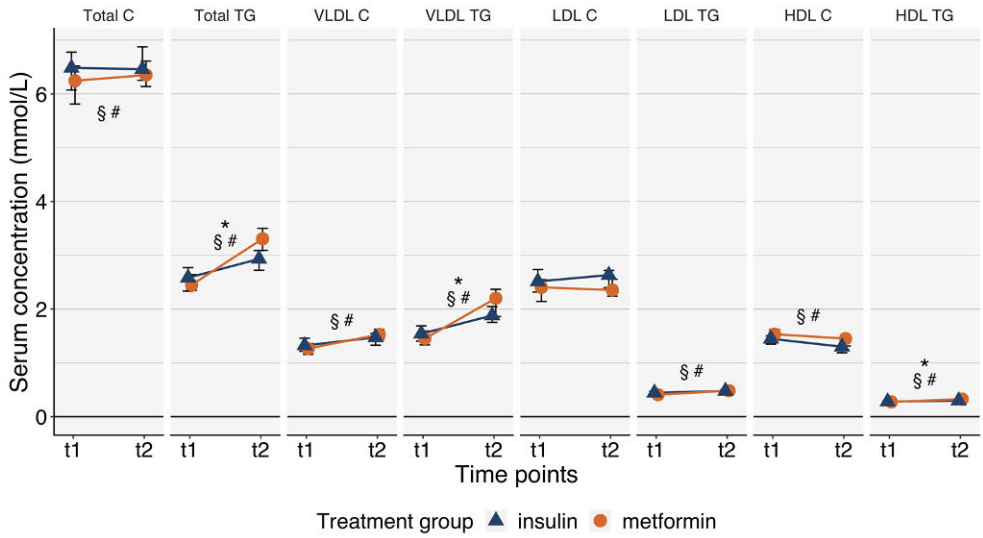

Figure 6. Median concentrations (± 95% confidence intervals) of maternal serum cholesterol (C) and triglycerides (TG) in total and in lipoproteins at baseline (t1) and at 36 gestational weeks (t2) are depicted in line graph. Statistical significance (p-value < 0.01) is denoted for changes within group (# metformin, § insulin) and for differences in median changes between groups (*). Metformin n = 99, insulin n = 91. Reproduced from original publication III, Figure 1.

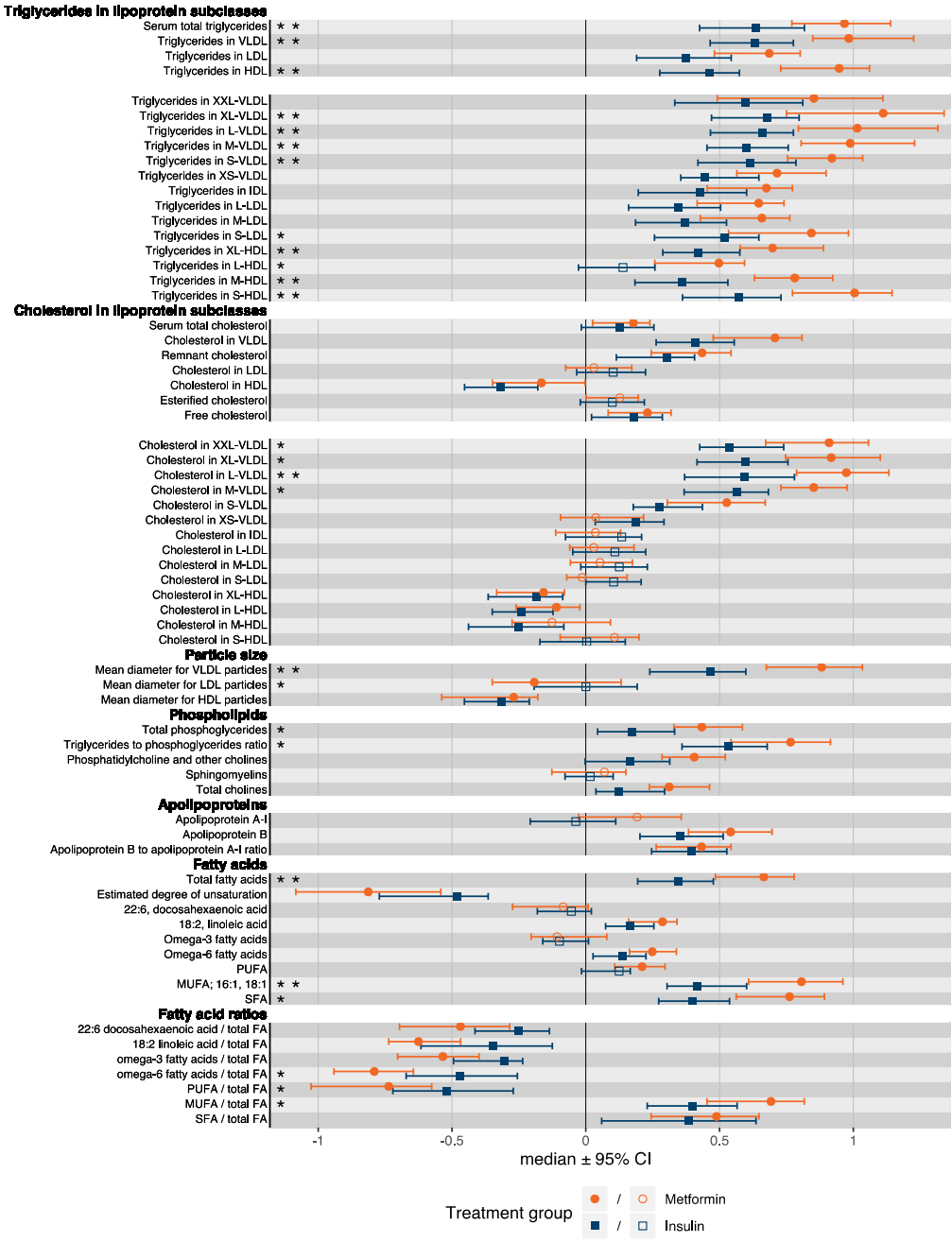

Figure 7. Impact of metformin and insulin on maternal serum lipidome. Median change scaled by baseline SD ± 95% CI. Closed squares / circles denote significant ($p < 0.01$) and open squares / circles non-significant change from baseline within group, * $p < 0.01$ for difference in change between groups, ** $p < 0.001$ for difference in change between groups. Metformin n = 95–99 (circles), insulin n = 89–91 (squares). CI: confidence intervals, FA: fatty acids, MUFA: monounsaturated FA, PUFA: polyunsaturated FA, SD: standard deviation, SFA: saturated FA. Reproduced from original publication III, Figure 2.

5.5.2 Association between the maternal serum lipidome and birth weight in patients requiring pharmacological treatment

At baseline, the maternal lipidome was associated with BW adjusted for gestational age (Q^2 = 4.66%), among the patients requiring pharmacological treatment. Total TG, TG in VLDL subclasses, IDL, LDL subclasses and in small HDL were positively related to BW in both treatment groups combined (Figure 8). VLDL cholesterol, cholesterol in all VLDL except in very small VLDL and remnant cholesterol were positively associated with BW, whereas cholesterol in medium HDL was inversely related to BW. Both apoB and the apoB to apoA-1 ratio were positively related to BW. The TG to phosphoglycerides ratio, total FA, MUFA and SFA were significantly associated with higher BW. Adjustment for pBMI, GWG or HbA1c had no drastic effects on these associations (Figure 8). Separate regression coefficients for metformin and insulin groups were calculated in case there was a significant ($p < 0.01$) interaction between the independent variable and the treatment. The positive associations between cholesterol in VLDL, cholesterol in medium and small VLDL, apoB and the apoB to apoA-1 ratio with BW were significant in the metformin but not in the insulin group (Figure 8). In addition, there was a significant positive association between LA, omega-6 FA and PUFA and BW only in the metformin group.

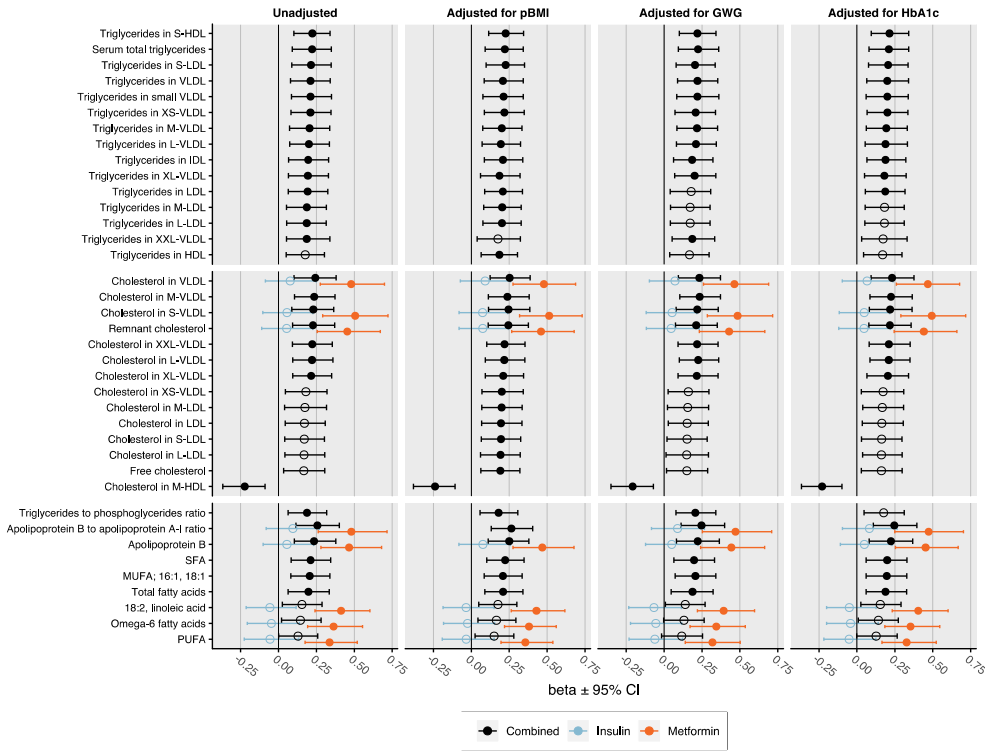

Figure 8. Associations between maternal serum baseline lipids and birth weight in unadjusted regression model and regression models adjusted for maternal pre-pregnancy BMI (pBMI), gestational weight gain (GWG) and glycated hemoglobin (HbA1c). Regression β-estimates (SD/SD) with 95% confidence intervals (CI) for significant ($p < 0.01$) associations between serum lipids at baseline and birth weight for both treatment groups combined (metformin and insulin). β-estimates for the groups are given individually, if there was a significant interaction between treatment groups and the association between independent and outcome variables. Closed circles denote significant ($p < 0.01$) associations. Birth weight was calculated as the deviation from the Finnish general population mean adjusted for gestation length. n = 204 (metformin: 104, insulin: 100) for the unadjusted analyses and analyses adjusted for pBMI and HbA1c, n = 203 (metformin: 103, insulin: 100) for the analyses adjusted for GWG. SD: standard deviation, MUFA: monounsaturated FA, PUFA: polyunsaturated FA, SFA: saturated FA.

Among the patients who required pharmacological treatment, the maternal lipidome was weakly associated with a risk of SGA ($Q^2 = 1.36\%$), but not LGA ($Q^2 < 0$). Logistic regression analyses showed that only higher levels of cholesterol in medium HDL were significantly associated with a higher SGA risk and this association remained relatively unchanged after adjustment for pBMI, GWG or HbA1c (Table 11). There were no significant interactions between the treatment groups and the associations between maternal lipids and SGA risk.

Table 11. Significant associations between maternal serum lipids and SGA risk.

	Unadjusted	**Adjusted for pBMI**	**Adjusted for GWG**	**Adjusted for HbA1c**
Baseline				
n	***204** (19/185)*	***204** (19/185)*	***203** (19/184)*	***204** (19/185)*
M-HDL cholesterol	2.0 [1.3; 3.3] *	2.0 [1.3; 3.3] *	2.0 [1.3; 3.3] *	2.1 [1.4; 3.4] *
36 gestational weeks				
n	***194** (20/174)*	***194** (20/174)*	***193** (20/174)*	***186** (19/167)*
Total cholesterol	0.52 [0.3; 0.78]	0.52 [0.30; 0.80]	0.51 [0.29; 0.78]	0.44 [0.25; 0.68] *
LDL cholesterol	0.46 [0.27; 0.71]	0.47 [0.27; 0.76]	0.46 [0.27; 0.75]	0.39 [0.23; 0.62] *
Esterified cholesterol	0.53 [0.31; 0.78]	0.53 [0.31; 0.8]	0.52 [0.3; 0.79]	0.45 [0.26; 0.69] *
Free cholesterol	0.5 [0.28; 0.77]	0.5 [0.28; 0.79]	0.49 [0.26; 0.78]	0.43 [0.23; 0.69] *
XS-VLDL cholesterol	0.46 [0.26; 0.71]	0.46 [0.26; 0.75]	0.46 [0.25; 0.74]	0.41 [0.24; 0.67] *
IDL cholesterol	0.47 [0.27; 0.71]	0.47 [0.28; 0.73]	0.46 [0.26; 0.74]	0.39 [0.23; 0.63] *
L-LDL cholesterol	0.46 [0.27; 0.72]	0.47 [0.27; 0.75]	0.46 [0.26; 0.74]	0.39 [0.23; 0.63] *
M-LDL cholesterol	0.46 [0.26; 0.72]	0.46 [0.27; 0.74]	0.46 [0.26; 0.74]	0.39 [0.22; 0.61] *
S-LDL cholesterol	0.47 [0.27; 0.72]	0.47 [0.27; 0.76]	0.47 [0.26; 0.74]	0.4 [0.23; 0.63] *
Sphingomyelins	0.56 [0.33; 0.85]	0.57 [0.33; 0.87]	0.55 [0.33; 0.86]	0.48 [0.29; 0.73] *

Logistic regression β-estimates (odds ratio / standard deviation) [95% confidence intervals] are given for unadjusted model and models adjusted for maternal pre-pregnancy BMI (pBMI), gestational weight gain (GWG) and glycated hemoglobin (HbA1c). Small for gestational age (SGA) was defined as birth weight < 10^{th} centile. The number of patients in each analysis (n) are given as total (SGA / non-SGA). Non-SGA was used as a reference. * $p < 0.01$.

The difference between metformin and insulin treatments regarding the association between selected maternal lipid concentrations at baseline and BW was further studied by stratifying the data into quartiles by maternal lipid concentrations and by comparing BW between the treatment groups within each lipid quartile (Table 12). The mothers who had the highest baseline VLDL cholesterol or apoB to apoA-1 ratio delivered heavier babies in the metformin group. In no other quartiles were there any significant differences between the groups.

Table 12. Birth weight centiles of neonates to metformin and insulin-treated GDM patients stratified by baseline maternal serum lipid quartiles. Modified from original publication III, Table 2.

	Q1	Q2	Q3	Q4
Total triglycerides				
Range (mmol/l)	0.91–2.05	2.06–2.52	2.53–3.02	3.03–5.13
Metformin	0.38 [0.31; 0.58] (29)	0.46 [0.31; 0.66] (29)	0.69 [0.54; 0.79] (26)	0.74 [0.58; 0.84] (20)
Insulin	0.42 [0.31; 0.62] (24)	0.60 [0.34; 0.76] (22)	0.67 [0.58; 0.88] (24)	0.54 [0.34; 0.69] (30)
p-value	0.50	0.31	0.96	0.061
VLDL cholesterol				
Range (mmol/l)	0.55–1.06	1.07–1.32	1.33–1.58	1.59–2.63
Metformin	0.38 [0.24; 0.56] (29)	0.48 [0.34; 0.58] (26)	0.6 [0.46; 0.79] (32)	0.79 [0.66; 0.86] (17)
Insulin	0.42 [0.29; 0.65] (23)	0.54 [0.34; 0.71] (26)	0.71 [0.58; 0.9] (18)	0.54 [0.34; 0.71] (33)
p-value	0.36	0.52	0.37	**0.014**[#]
VLDL triglycerides				
Range (mmol/l)	0.41–1.17	1.18–1.52	1.53–1.93	1.94–3.86
Metformin	0.42 [0.31; 0.66] (29)	0.48 [0.34; 0.62] (26)	0.66 [0.46; 0.76] (27)	0.76 [0.58; 0.84] (22)
Insulin	0.42 [0.29; 0.54] (23)	0.62 [0.44; 0.76] (26)	0.67 [0.46; 0.88] (22)	0.54 [0.42; 0.73] (29)
p-value	0.80	0.30	0.64	0.085
ApoB to apoA-1 ratio				
Range (ratio)	0.36–0.67	0.68–0.79	0.80–0.90	0.91–1.34
Metformin	0.34 [0.18; 0.42] (31)	0.54 [0.46; 0.71] (26)	0.62 [0.42; 0.76] (29)	0.76 [0.60; 0.96] (18)
Insulin	0.42 [0.27; 0.66] (22)	0.58 [0.48; 0.76] (23)	0.64 [0.46; 0.76] (22)	0.66 [0.38; 0.76] (33)
p-value	0.32	0.76	0.92	**0.026**[#]

Data is reported as median birth weight percentile [95% confidence interval] (n) in quartiles (Q1–Q4) of each lipid (total triglycerides, VLDL cholesterol, VLDL triglycerides, apolipoprotein B to A-1 ratio). Birth weight was calculated as the deviation from the Finnish general population mean adjusted for gestation length. p-value is given for the Mann-Whitney U test for comparisons of birth weights between treatment groups in each lipid quartile. # The differences between metformin and insulin groups in Q4 were significant ($0.01 < p < 0.05$), also after adjustment (ANCOVA) separately for pre-pregnancy BMI, maternal gestational weight gain and baseline HbA1c.

The association between the maternal lipidome and BW was not as strong as at baseline at 36 gw (Q^2 = 1.48%) in the combined metformin and insulin groups. Analyzed individually, only TG in total LDL, TG in medium and large LDL and cholesterol in small and very small VLDL were positively related to BW (Figure 9).

Adjustment for pBMI, GWG or HbA1c had only a modest effect on these associations (Figure 9). When separate regression coefficients for the metformin and the insulin group were taken into consideration, the associations between TG IDL, cholesterol in small VLDL and BW were significant in the metformin but not in the insulin group. There was a significant association between TG in small and very small VLDL, cholesterol in VLDL, remnant cholesterol, apoB, apoB to apoA-1 ratio, LA, omega-6 FA and total FA only in the metformin group (Figure 9).

In the pharmacological treatment group, the serum lipidome at 36 gw was weakly associated with SGA ($Q^2 = 0.60\%$) but not with LGA ($Q^2 < 0$) risk. None of the lipids was individually significantly associated with the risk of SGA. After adjusting for maternal HbA1c at 36 gw there were significant inverse associations between total cholesterol, cholesterol in very small VLDL, IDL cholesterol, cholesterol in all LDL subclasses, esterified cholesterol, free cholesterol and sphingomyelins and the risk of SGA (Table 11).

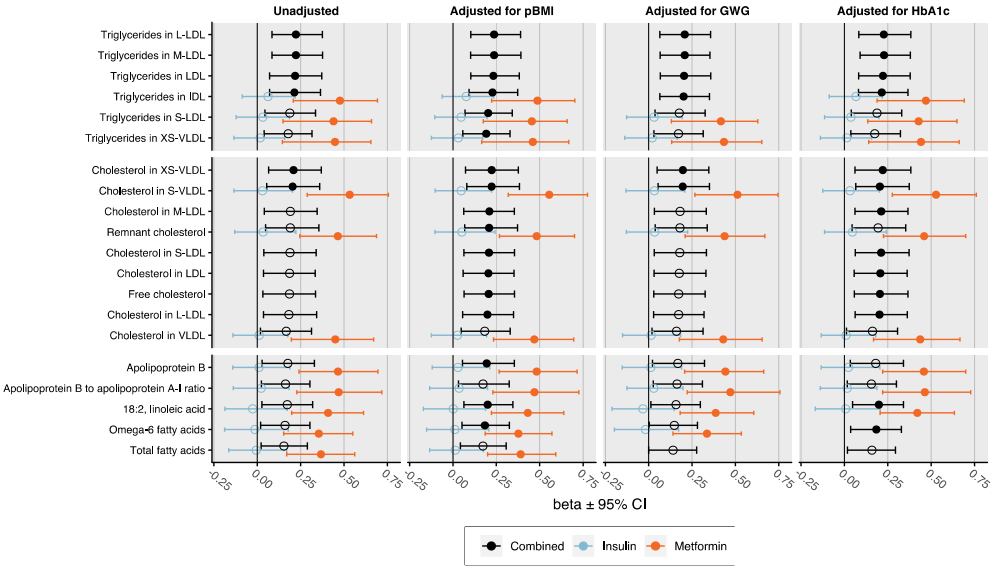

Figure 9. Associations between maternal serum lipids at 36 gestational weeks and birth weight in unadjusted regression model and regression models adjusted for maternal pre-pregnancy BMI (pBMI), gestational weight gain (GWG) and glycated hemoglobin (HbA1c). Regression β-estimates (SD/SD) with 95% confidence intervals (CI) for significant ($p < 0.01$) associations between serum lipids at baseline and birth weight for both treatment groups combined (metformin and insulin). β-estimates for the groups are given individually, if there was a significant interaction between treatment groups and the association between independent and outcome variables. Closed circles denote significant ($p < 0.01$) associations. Birth weight was calculated as the deviation from the Finnish general population mean adjusted for gestation length. n = 194 (metformin: 96, insulin: 98) for the unadjusted analyses and analyses adjusted for pBMI, the corresponding n-values for the analyses adjusted for GWG and HbA1c are combined: 193, metformin: 95, insulin 98 and combined: 186, metformin: 93, insulin: 93.

5.5.3 Association between maternal serum lipidome and other clinical outcome variables than birth weight

At baseline, the maternal serum lipidome was associated with total GWG (Q^2 = 3.33%) and length of gestation at delivery (Q^2 = 1.14%) by multivariate PLS regression. Similarly, at 36 gw the maternal serum lipidome was associated with total GWG (Q^2 = 3.20%), but not with length of gestation at delivery. The maternal serum lipidome was neither at baseline nor at 36 gw associated with hypertensive disorders, risk of cesarean delivery, NICU admission or neonatal I.V. glucose administration ($Q^2 < 0$). Of note, in the PLS models on maternal serum lipidome and GWG at baseline and at 36 gw, the first three components of the PLS decomposition were predictive for the outcome, whereas only the association between the first PLS component and the outcome was significant regarding the other outcome variables.

The significant ($p < 0.01$) associations between maternal serum metabolites and GWG are presented in Table 13. At baseline, only the ratio of MUFA to total FA was inversely related to GWG. This association was attenuated when the model was adjusted for pBMI but not when adjusted for HbA1c. At 36 gw, TG in large HDL and mean diameter of HDL were positively related to GWG. The association between TG in large HDL and GWG was not affected by adjustment for HbA1c. The ratio of SFA to total FA was significantly and positively related to GWG only after adjustment for pBMI. Treatment (metformin or insulin) did not interact significantly with the associations between maternal lipids and GWG.

Regression analyses revealed no significant associations between individual lipids at baseline and length of gestation at delivery.

Table 13. Significant associations between maternal serum lipids and gestational weight gain.

	Unadjusted	**Adjusted for pBMI**	**Adjusted for HbA1c**
Baseline			
Ratio of MUFA to total FA	-0.2 [-0.33; -0.074] *	-0.15 [-0.27; -0.023]	-0.2 [-0.33; -0.074] *
36 gestational weeks			
Triglycerides in large HDL	0.21 [0.08; 0.35] *	0.17 [0.049; 0.3]	0.19 [0.058; 0.32] *
Mean diameter of HDL particles	0.19 [0.044; 0.34] *	0.17 [0.04; 0.3]	0.17 [0.033; 0.32]
Ratio of SFA to total FA	0.18 [0.04; 0.32]	0.2 [0.078; 0.33] *	0.12 [-0.033; 0.27]

Data is reported as β-estimates (SD/SD) with 95% confidence intervals for unadjusted regression models and models adjusted for pre-pregnancy BMI (pBMI) and maternal glycated hemoglobin (HbA1c). * $p < 0.01$. SD: standard deviation, MUFA: monounsaturated fatty acids, FA: fatty acids, SFA: saturated fatty acids.

5.6 Cord serum metabolome (Study IV)

5.6.1 Effect of maternal metformin and insulin treatments on the cord serum metabolome

The cord serum metabolites were compared between all three groups (diet, insulin and metformin). The only statistically significant difference at a level of $p < 0.01$ was higher alanine in the metformin group (0.53 mmol/l) than in the insulin (0.45 mmol/l, $p < 0.001$) or the diet group (0.46 mmol/l, $p < 0.0001$) (Figure 10).

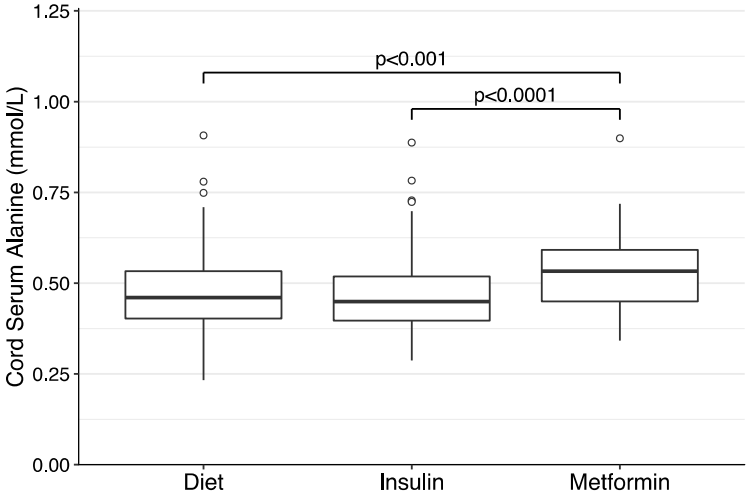

Figure 10. Boxplot representation of the distributions of cord serum alanine concentration in groups treated with diet, insulin and metformin. Interquartile ranges with medians are marked with boxes and whiskers denote range. Outliers, defined as being further away than 1.5 times the interquartile range from the median, are marked with circles. P-values are given for differences in the Dwass-Steele test and only significant values are shown, Submitted manuscript IV, Figure 1.

5.6.2 Associations between cord serum metabolites and birth weight

The associations between cord serum metabolites and BW were first calculated in the whole study population (diet, metformin and insulin groups). Unadjusted regression models showed that the ratio of TG to phosphoglycerides and the average VLDL diameter were inversely related to BW (Figure 11). The proportion of omega-6 FA of total FA, the ratio of omega-6 to omega-3 FA and the ratio of PUFA to MUFA were positively associated with BW. Accordingly, the proportions on MUFA and omega-3 FA, DHA and omega-3 FA were inversely related to BW. Of the amino acids, only histidine was significantly related to BW. In addition, two ketones, 3-

hydroxybutyrate and acetone, were positively associated with BW. When adjusted for pBMI, GWG or maternal baseline HbA1c, also cholesterol in very large HDL and the average HDL diameter were significantly associated with BW. The positive association between total lipids in very large HDL and BW was significant only after adjustment for HbA1c and the positive association between the proportion of PUFA and BW when adjusted for pBMI. The degree of unsaturation was significantly associated with higher BW after adjustment for pBMI or GWG.

The treatment group interacted significantly ($p < 0.05$) only with the association between the omega-6 to omega-3 FA ratio and BW. The association was significant in the insulin group (0.34; CI: 0.12, 0.56 SD/SD), but not in the metformin (0.22; CI: -0.010, 0.45 SD/SD) or the diet group (0.045; CI: -0.12, 0.21 SD/SD). Adjustment for mode of delivery did not notably affect the results.

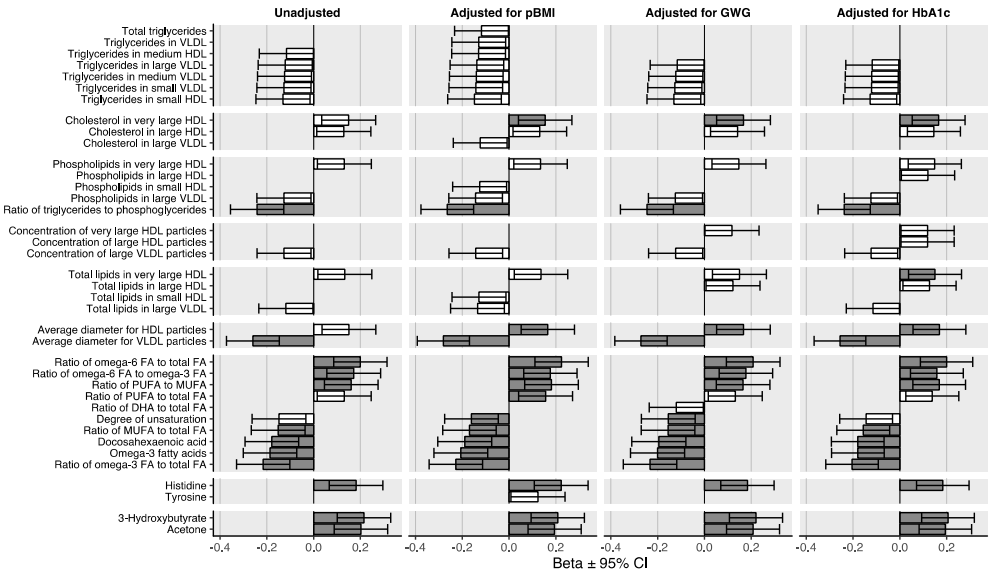

Figure 11. Significant associations between cord serum metabolites and birth weight. β-estimates (SD/SD) with 95% confidence intervals (CI) are shown for unadjusted linear regression and regression models adjusted for maternal pre-pregnancy BMI (pBMI), gestational weight gain (GWG) and maternal glycated hemoglobin (HbA1c). Birth weight was expressed in SD units, i.e., deviation from the Finnish population mean BW adjusted for gestation duration. White bars denote $p < 0.05$ and gray bars $p < 0.01$. Only associations with $p < 0.05$ are shown. SD: standard deviation, FA: fatty acids, DHA: docosahexaenoic acid, MUFA: monounsaturated FA, PUFA: polyunsaturated FA, SGA: saturated FA. Submitted manuscript IV, Figure 2.

6 Discussion

Treatment of GDM is beneficial (Crowther *et al.*, 2005; Langer *et al.*, 2005a; Landon *et al.*, 2009). Still, the outcomes of GDM pregnancies are inferior to non-GDM pregnancies (Reinders *et al.*, 2020), and more work is required to optimize GDM treatment and to reduce maternal and neonatal risks.

Metformin treatment of GDM is more affordable and comfortable for the patient than injectable insulin. Metformin is also comparable, if not superior, to insulin regarding short-term perinatal outcomes (Butalia *et al.*, 2017; Farrar *et al.*, 2017b). In this study the effects of metformin treatment on maternal and neonatal metabolome were assessed with the aim of identifying metabolic and inflammatory risk factors that indicate antihyperglycemic pharmacological treatment. The associations between these factors and adverse perinatal outcomes in GDM were studied to evaluate the metabolic safety of metformin.

The main novel findings were: First, the concentration of serum alanine, total TG, VLDL TG and total FA increased more in the metformin-treated patients than the insulin-treated patients. Second, the associations between maternal VLDL cholesterol and the apoB to apoA-1 ratio and BW differed between the metformin and insulin groups. Third, compared to insulin, the metformin treatment of GDM had no effects on neonatal metabolome except the higher cord serum alanine.

6.1 Methodological and ethical considerations and strengths and limitations of the study

The study population was derived from a previous randomized controlled trial (Tertti *et al.*, 2013). A placebo group was not possible for ethical reasons. Hence, as a reference group, patients who met the same inclusion and exclusion criteria, but who achieved appropriate glycemic goals without pharmacological interventions, were included. The original trial was approved by the local ethics committee with appropriate drug regulatory agencies as described in section 4.1. In this secondary analysis no new samples were collected in addition to those that had already been collected and the subjects were aware that blood samples could be studied later.

The strengths of the study are the prospective longitudinal design, treatment allocation to either metformin or insulin by randomization, the large sample size and

the use of validated metabolome analysis method. There are no previous studies comparing the effects of metformin versus insulin on the maternal serum or neonatal cord blood serum metabolome in pregnancies complicated by GDM.

The use of data from a randomized trial reduces the risk of selection bias for each treatment and the groups were comparable at baseline. This makes the evaluation of drug effects between the groups reliable. However, because there was no placebo group, evaluation of metformin and insulin effects individually was challenging. Having had serum samples also from the diet group at 36 gw would have helped interpretation of the results.

The study population is ethnically homogenous, rather large and the overall glycemic control was good. Although this benefits the interpretation of the data, the results may not be generalized to populations of other ethnic background or with poor glycemic control. For a more detailed picture of the glycemic control of the study subjects, CGM data would be needed.

NMR was applied to analyze the targeted maternal serum metabolome. This method is fast and has good reproducibility (Soininen *et al.*, 2009). All serum samples were stored at -70°C prior to analyses and analyzed concurrently. High throughput and reliable data may make NMR usable for biochemical analyses also in the clinical settings in the future. A drawback of NMR is, however, its limited capacity to measure metabolites present in very low concentrations.

To analyze the large metabolome datasets, both univariate and multivariate methods were used. PLS and PLS-DA are suitable methods for intercorrelated data (Worley *et al.*, 2015), such as metabolome datasets, and were used in our study to estimate the associations between the maternal serum lipidome and measures of BW. Stricter thresholds for p-values were applied and cross-validation was used in the multivariate models to decrease the risk of type I error, i.e., false positive.

Since this was a secondary analysis, we could not affect the number of patients in the study. The original randomized trial was powered to prove non-inferiority of metformin compared to insulin, regarding the BW variable. Hence, the present study was underpowered to study associations in detail between single maternal metabolites and some of the infrequent perinatal outcomes. Moreover, the indications for inductions of labor and for cesarean deliveries were not recorded, which complicates the interpretation of the data.

6.2 Antihyperglycemic treatment need is poorly reflected by maternal serum metabolome or inflammatory markers

At the time of the GDM diagnosis, there were only few differences between the patients requiring and not requiring pharmacological treatment. Maternal serum

glutamine was higher ($p = 0.009$) and glucose lower ($p < 0.0001$) in the diet group and, at a p-value threshold of 0.05, also TG in small VLDL was marginally lower in the diet group ($p = 0.046$).

The lower glutamine in the diet groups fits the notion that outside pregnancy glutamine is inversely associated with an increased risk of T2DM (Cheng *et al.*, 2012; Guasch-Ferré *et al.*, 2016). It is however unclear whether glutamine is altered in GDM (Butte *et al.*, 1999; Cetin *et al.*, 2005; Pappa *et al.*, 2007; Rahimi *et al.*, 2017; Mokkala *et al.*, 2020b).

Comparing the maternal metabolome between the highest and lowest fasting glucose deciles of the HAPO cohort, several amino acids (including alanine, proline, glutamine/glutamate, arginine and leucine/isoleucine), TG and 3-hydroxybutyrate were higher in the high glucose group (Scholtens *et al.*, 2014). These results are similar to ours, although the mean fasting plasma glucose values were quite different from our study population: in the HAPO the OGTT fasting plasma glucose values were 5.3 mmol/l (94.9 mg/dl) and 3.8 mmol/l (68.5 mg/dl), in the high and low glucose groups, respectively. (In our study the fasting glucose values were 5.38 mmol/l in the diet and 5.54 mmol/l in the combined pharmacological treatment group.)

Perhaps less stringent inclusion criteria to include also milder forms of GDM would have yielded different results, but our data show that at the time of diagnosis the maternal metabolome is unlikely to distinguish patients who will require pharmacological treatment from those who will not. An explanation might be that there are behavioral factors, e.g., adherence to medical nutrition therapy, that contribute to the need of pharmacological therapy, but are unrelated to the maternal metabolome. In a large metabolomic analysis it was found that maternal acylcarnitines, in particular, measured at 1 h after glucose load but not at fasting, were, mostly positively, related to glucose values (Scholtens *et al.*, 2016). Thus, analysis of the maternal metabolome also after an oral glucose load might improve identification of patients who will need pharmacological treatment.

6.3 Metformin treatment compared to insulin causes distinct alterations in the maternal metabolome

6.3.1 Increased alanine concentration

In previous population studies most amino acids increased from the second to the third trimester, except valine which decreased clearly (Lindsay *et al.*, 2015; Wang *et al.*, 2016). These changes were more pronounced in our study among women with

GDM, who also had an increase in glycine, in contrast to a decrease observed in healthy pregnancies (Lindsay *et al.*, 2015).

The changes in the concentrations of individual amino acids were similar in the metformin and insulin groups with few exceptions. Most noticeably, metformin led to a greater increase in maternal serum alanine than insulin. An increase in alanine in response to metformin has been demonstrated earlier in non-pregnant subjects both with and without diabetes (Nattrass *et al.*, 1977; Preiss *et al.*, 2016; Eppinga *et al.*, 2017).

The metformin-related increase in alanine may be due to suppression of gluconeogenesis in the liver (Hundal *et al.*, 2000), which causes accumulation of gluconeogenic substrates, such as alanine and lactate. Metformin has also been shown to suppress glucagon signaling in the liver (Miller *et al.*, 2013). The paradigm that metformin actually affects gluconeogenesis has, nevertheless, been challenged and instead metformin might increase non-oxidative glucose disposal with a simultaneous rise in serum glucagon (Gormsen *et al.*, 2019). The relationship between glucagon and alanine is inverse rather than positive (Wewer Albrechtsen *et al.*, 2018), and hence it is unlikely that the effect of metformin on alanine could be explained by increased glucagon. Nonetheless, increases in non-oxidative glucose disposal could lead to increases in alanine and lactate, as well.

The observed increase in isoleucine in the metformin group compared to the insulin group seems paradoxical. Previously it has been reported that isoleucine and leucine are raised in T2DM patients on metformin (Safai *et al.*, 2018) and increased in response to metformin in insulin resistant, but not insulin sensitive patients without T2DM (Walford *et al.*, 2013). According to earlier studies insulin reduces leucine concentrations (Castellino *et al.*, 1987), while metformin has no effects on amino acid metabolism (Tessari *et al.*, 1994). The greater increase of isoleucine in the metformin group observed in our data could thus be due to insulin attenuating the increase of leucine and isoleucine.

6.3.2 Increased triglyceride and very low-density lipoproteins

The changes in maternal lipids during the last trimester of pregnancy were generally parallel in the insulin and metformin groups. Increased insulin resistance and estrogen cause increased VLDL production and decreased clearance (Herrera *et al.*, 2016). Consequently, several studies have reported a progressive increase in circulating VLDL in pregnancy (Jimenez *et al.*, 1988; Montelongo *et al.*, 1992; Wang *et al.*, 2016; Mills *et al.*, 2019).

In our study LDL TG increased also, but LDL cholesterol did not. The LDL TG increase is probably due to increased CETP activity (Silliman *et al.*, 1993; Iglesias

et al., 1994) as has been reported previously (Alvarez *et al.*, 1996; Wang *et al.*, 2016; Mills *et al.*, 2019). In previous studies, LDL cholesterol increased from the second to the third trimester (Jimenez *et al.*, 1988; Alvarez *et al.*, 1996; Wang *et al.*, 2016), although not in GDM (Montelongo *et al.*, 1992; Barrett *et al.*, 2013a).

Similarly as TG in VLDL and LDL, also HDL TG increased, in agreement with previous findings (Montelongo *et al.*, 1992; Alvarez *et al.*, 1996; Wang *et al.*, 2016). The changes in HDL cholesterol are, however, more complex: HDL2 cholesterol decreases and HDL3 cholesterol increases during the last half of pregnancy (Alvarez *et al.*, 1996; Wang *et al.*, 2016; Mills *et al.*, 2019). This is in accordance with our data, where cholesterol in the larger HDL particles tended to decrease and cholesterol in small HDL did not change in either treatment group. Total HDL cholesterol did, however, decrease. We also observed a small decrease in mean HDL particle size which may reflect a change in the lipid content or in HDL subclass distribution.

In accordance with other studies, the mean VLDL size increased (Wang *et al.*, 2016; Mills *et al.*, 2019), probably due to increased TG content.

In a large randomized trial comparing metformin and insulin in GDM (the MiG trial), maternal TG increased significantly more in the metformin than the insulin group (Barrett *et al.*, 2013a). A smaller trial showed a marginally and non-significantly higher TG concentration among women treated with metformin compared to insulin (Zawiejska *et al.*, 2016). We extend these findings by showing that the increase in TG is evident in virtually all VLDL and HDL lipoprotein particle subclasses. Of the LDL fractions, this difference was significant only in small LDL particles.

There was also a higher increase in VLDL cholesterol in the metformin than the insulin group, probably due to an overall greater increase in VLDL particle concentrations.

Our study did not include a placebo group and it is thus not reasonable to expect robust discrimination of whether the observed differences between the two treatments are attributable to metformin or insulin. As suggested by Barrett *et al.*, women randomized to metformin treatment might have substituted more carbohydrates with dietary fat in order to stay normoglycemic and avoid additional insulin (Barrett *et al.*, 2013a).

There are several reasons, however, to assume that the difference in our study regarding TG, especially in VLDL subfractions, is attributable to insulin treatment. First, in a meta-analysis of T2DM patients metformin did not affect plasma TG (Wulffelé *et al.*, 2004). Second, a placebo-controlled study showed that metformin did not affect maternal TG during pregnancy (Chiswick *et al.*, 2015). And third, metformin does not affect hepatic VLDL TG secretion in T2DM (Gormsen *et al.*, 2018), while insulin suppresses VLDL TG secretion (Sørensen *et al.*, 2011).

Total FA increased in both treatment groups and this increase was mostly driven by SFA and MUFA. The proportion of MUFA, omega-3 FA and omega-6 FA of total FA decreased. These findings are in agreement with a previous large cohort study (Wang *et al.*, 2016).

The increase in total FA, SFA and MUFA was greater and the proportions of omega-6 FA, PUFA and MUFA of total FA decreased more in the metformin group. In contrast, a previous small randomized trial of non-pregnant subjects showed that metformin did not affect the serum FA profile (Rodríguez *et al.*, 2004). The difference may be explained by pregnancy and GDM. In addition, in our study metformin treatment was compared to insulin, not placebo. Metformin may cause gastrointestinal side effects and, as discussed above, the patients on metformin may have additional incentive to remain normoglycemic and switch dietary carbohydrates to fat. The differences in circulating FA could reflect differences in dietary patterns, or altered FA metabolism during GDM pregnancy.

Compared to insulin, metformin treatment led to more atherogenic lipid profile in pregnant women with GDM. As the dyslipidemia resolves rather quickly postpartum (Barrett *et al.*, 2013a), it seems unlikely that metformin would increase the risk of major long-term adverse cardiovascular outcomes for the mothers. On the other hand, insulin is associated with higher GWG and weight retention postpartum (Rowan *et al.*, 2008). The effects of metformin or insulin treatment on maternal long-term outcomes and the importance of maternal serum lipidome in this regard warrant further follow-up studies.

6.3.3 Effect of metformin on inflammatory markers

Of the four measured inflammatory markers, hsCRP decreased, IL-6 and GlycA increased and MMP-8 remained unchanged during the last third of pregnancy in the patients treated with either metformin or insulin. The decrease in CRP and increase in IL-6 and GlycA are in line with previous data on non-diabetic women (Christian *et al.*, 2014; Wang *et al.*, 2016). The changes in serum MMP-8 have not been previously studied in pregnancy. In general, the concentrations of CRP and IL-6 correlate positively, since IL-6 promotes CRP secretion in the liver, but the direction of change was opposite between hsCRP and IL-6. Compared to insulin, metformin affected neither hsCRP, IL-6 nor MMP-8. Maternal CRP was studied in a subsample of the MiG trial and the authors reported, in agreement with our data, that metformin compared to insulin did not affect CRP (Barrett *et al.*, 2013a). They also found that CRP remained unchanged from baseline to 36 gw, whereas we observed a decrease. This discrepancy may be related to higher BMI in the MiG trial compared to our study, since hsCRP is related to maternal BMI (Kuzmicki *et al.*, 2008; Christian *et al.*, 2014), or to a higher baseline CRP in the MiG trial.

GlycA increased significantly in both groups, but more in the metformin group. It is elevated in pregnancies complicated by GDM (White *et al.*, 2017; Mokkala *et al.*, 2020b), and in early pregnancy GlycA is positively related to insulin resistance, TG and LDL cholesterol (Mokkala *et al.*, 2017). According to one study, four months treatment with metformin outside pregnancy did not affect serum GlycA (Eppinga *et al.*, 2017), but in another smaller study GlycA was lower among metformin-treated patients with T2DM compared to untreated patients (Huo *et al.*, 2009). GlycA is a composite marker of several serum proteins, such as α-1-acid glycoprotein, haptoglobin, α-1-antitrypsin, α-1-antichymotrypsin and transferrin (Bell *et al.*, 1987), which have their own trajectories during pregnancy (Honda *et al.*, 1990; Larsson *et al.*, 2008). We found that GlycA correlated positively with HbA1c and fasting C-peptide at baseline, but not with HbA1c at 36 gw. Hence, the composition of GlycA may be different in different stages of pregnancy, but also GDM treatment may affect single components of GlycA. We did not find significant associations between late pregnancy GlycA and perinatal outcomes; the significance of elevated GlycA in the third trimester of pregnancy needs further evaluation.

6.4 Changes in IGFBP-1 phosphoisoforms and association with birth weight

According to previous studies IGFBP-1 concentrations increase in early pregnancy and remain relatively unchanged thereafter (Clapp *et al.*, 2004; Larsson *et al.*, 2013). In contrast, in late pregnancy complicated by GDM we observed a clear increase in all IGFBP-1 phosphoisoform serum concentrations. In the metformin group, the increase in non-pIGFBP-1 was significantly higher than in the insulin group and a similar trend was also seen for low-pIGFBP-1. In PCOS, metformin increases IGFBP-1 (De Leo *et al.*, 2000; Jakubowicz *et al.*, 2001; Pawelczyk *et al.*, 2004). Insulin inhibits IGFBP-1 production, and the increase in IGFBP-1 concentration in the metformin group could be due to improved insulin sensitivity and, consequently, to lower plasma insulin levels. However, also a direct effect of metformin on IGFBP-1 production has been demonstrated in vitro in malignant endometrial cells (Xie *et al.*, 2014). Moreover, as shown in patients with breast cancer, the effects of metformin treatment on IGFBP-1 are dependent on the patient's BMI with greater increases in overweight patients (DeCensi *et al.*, 2014).

While non-pIGFBP-1 increased more in the metformin group than the insulin group, there were no differences in high-pIGFBP-1, which was the most abundant phosphoisoform. Previously both high and low phosphorylated isoforms of IGFBP-1 have been reported to be higher in pregnant women with T1DM than in nondiabetic controls (Gibson *et al.*, 1999). IGFBP-1 is inversely related to maternal insulin resistance in pregnancy (Ramirez *et al.*, 2014) and the association seems to be related

to non-pIGFBP-1 rather than the phosphorylated form (Mokkala *et al.*, 2020a). In a small study, high-pIGFBP-1 in T1DM was inversely related to BW, but low-pIGFBP-1 was not (Gibson *et al.*, 1999). Similarly, in a population cohort IGFBP-1 was inversely related to BW (Åsvold *et al.*, 2011). Hence, it seems that maternal IGFBP-1 is inversely related to BW, although there may be differences between uncomplicated pregnancies and pregnancies complicated by diabetes.

In our data only the non-pIGFBP-1 isoform at baseline was inversely related to BW. The reason for the lack of an association between IGFBP-1 at h36 and BW may be initiation of GDM treatment, which may have affected IGFBP-1 concentrations as well as the risk of fetal overgrowth.

6.5 Association of metformin exposure with cord serum alanine and lipids

Changes in maternal serum lipid profiles in late pregnancy were different between metformin and insulin-treated patients. Thus, it was unexpected to see essentially similar cord serum lipidome in the metformin, insulin and diet-treated patients. Previously the MiG trial showed that cord plasma TG, HDL cholesterol and LDL cholesterol are unaffected by metformin treatment compared to insulin (Barrett *et al.*, 2013a). We confirm these findings and demonstrate in a larger dataset that neither metformin nor insulin treatment of GDM affects the cord serum lipidome. It was also reassuring to find similar concentrations of cord serum lactate and ketone bodies in both treatment groups. Although the clinical characteristics of the diet group were not identical to those on pharmacological treatment, there were very few differences in maternal baseline or outcome variables. Thus, it is justified to use this diet group as a reference when comparing the cord serum metabolome of neonates to mothers treated with metformin and insulin.

While the lipidome was not affected, cord serum alanine was significantly higher in the offspring of women who were treated with metformin for GDM than women treated with insulin or diet only. A similar increase was also observed in the maternal serum alanine in the metformin group. Increased cord alanine has been previously reported in GDM (Cetin *et al.*, 2005; Dani *et al.*, 2014), but the effects of metformin treatment on cord alanine have not been studied previously.

The neonatal alanine could increase through three different mechanisms. First, high maternal serum alanine could result in increased placental transfer because of an increased concentration gradient. Accordingly, maternal and neonatal alanine concentrations correlate strongly, although less so in patients with GDM (Cetin *et al.*, 2005). Second, metformin could, in theory, augment the placental transfer of alanine. Contrariwise, it has been shown that inhibition of mTOR signaling downregulates system A and system L amino acid transporters (Rosario *et al.*, 2013)

and metformin inhibits mTOR (Kalender *et al.*, 2010). Third, metformin – which crosses the placenta (Vanky *et al.*, 2005; Tertti *et al.*, 2010) – could directly alter fetal metabolism, leading to accumulation of alanine.

As reviewed above, the exact mechanism of action of metformin is uncertain and consequently the effects of metformin in the fetus are not fully known. Nor are the regulation and role of endogenous glucose production in the human fetus fully understood (Girard, 1986). It has been proposed that metformin could act via the intestinal microbiota (Vallianou *et al.*, 2019), that is absent in the fetus. Hence, the effects of metformin on glucose metabolism in the fetus, if any, could differ from the effects in adults. Despite the higher alanine concentrations, we did not observe changes in other substrates of gluconeogenesis, lactate, pyruvate, glycerol and glutamine. It seems therefore unlikely that metformin causes major alterations in the fetal metabolism in late pregnancy.

Thus, we demonstrated a clear increase in neonatal alanine in response to maternal metformin treatment. The possible long-term implications of this finding need to be evaluated in further studies.

6.6 Associations between maternal and neonatal metabolomes and birth weight

Maternal glucose, lipids and amino acids are important determinants of fetal growth (Freinkel, 1980; HAPO Study Cooperative Research Group *et al.*, 2008). In the present study, we examined the associations between maternal metabolites and BW at different stages of pregnancy. Our population consists only of patients with GDM and the associations may differ from those in healthy pregnancy (Schaefer-Graf *et al.*, 2008, 2011).

6.6.1 Maternal serum metabolome

Glucose

Strong associations between maternal fasting glucose and BW and LGA risk at baseline were found, but these associations were attenuated at 36 gw. This may suggest that glycemic control is not as an important factor for fetal growth in late pregnancy as in earlier pregnancy, or that once hyperglycemia was under better control the reduced variation in glucose values led to loss of any evident association. Early pregnancy hyperglycemia may be more important than currently assumed for accelerating fetal growth through induced fetal hyperinsulinemia (Desoye *et al.*, 2016). The diurnal glucose patterns related to LGA are also different in each trimester, and they might not be reflected in overnight fasting glucose values, which

may hinder the ability of this measure to predict fetal growth (Law *et al.*, 2015, 2019).

Amino acids

Of the amino acids, only baseline leucine was related positively to BW. In the HAPO population, leucine/isoleucine was positively related to BW 1 h after glucose load, but not at fasting (Kadakia *et al.*, 2019a). The association was attenuated after adjustment for maternal BMI or OGTT 1 h glucose. Accordingly, in a smaller sample of diet treated GDM patients fasting leucine and isoleucine were positively correlated to BW, but this association was not significant after adjustment for fasting plasma glucose and pre-pregnancy body weight (Metzger, 1991). Leucine/isoleucine is shown to be inversely related to maternal insulin sensitivity but not BMI (Sandler *et al.*, 2017). Hence, it seems that BCAA leucine and isoleucine may not be independently strong promoters of fetal growth but are rather indicators of maternal insulin resistance.

Later, at 36 gw, alanine was associated positively with BW, although this association was not significant with a more conservative threshold of $p < 0.01$ or after FDR-adjustment. Separately, this association was significant for the patients on metformin but not on insulin treatment. There was also a significant association between alanine and HbA1c at 36 gw only in the metformin group. In the HAPO studies, similar to leucine/isoleucine, alanine at 1 h but not at fasting was positively related to BW (Kadakia *et al.*, 2019a). Alanine was also inversely associated with maternal insulin sensitivity, and this association persisted after adjustments for maternal BMI or glucose (Sandler *et al.*, 2017). Alanine is a major gluconeogenic precursor and could, in theory, promote fetal growth also via gluconeogenesis. However, based on previous studies (Hellmuth *et al.*, 2017c; Kadakia *et al.*, 2018, 2019b; Lu *et al.*, 2018; Patel *et al.*, 2018) and our data, alanine in cord blood is not associated with BW. Thus, metformin probably promotes alanine accumulation in the more insulin resistant GDM patients, but might not independently cause accelerated fetal growth.

Lipids

There is an association between maternal TG and BW, although it is modified by maternal obesity and GDM status (Schaefer-Graf *et al.*, 2008, 2011; Misra *et al.*, 2011; Geraghty *et al.*, 2016; Kadakia *et al.*, 2019a). We studied this association in several lipoprotein subclasses and found significant associations between BW and TG at baseline in VLDL, IDL and LDL, but only in small HDL particles. At 36 gw only IDL TG and large and medium LDL TG were significantly related to BW.

VLDL and LDL transfer lipids to tissues and organs, such as the placenta, while HDL transfers lipids in the opposite direction. Our data fits this pattern and provides a mechanistic explanation for the ability of TG to foster fetal growth, at least in a subset of patients. A Mendelian randomization analysis did not, however, support causal association between TG and BW (Tyrrell *et al.*, 2016). Rather, increased TG could be a bystander of maternal obesity (Sandler *et al.*, 2017), which could accelerate fetal growth via other mechanisms.

At 36 gestational weeks the associations between TG and BW were less robust, possibly due to intervention or to a decreased impact of serum TG on BW in late pregnancy.

Among different lipoprotein subclasses, the positive associations between cholesterol and BW were strongest regarding the VLDL lipoproteins. Of the HDL subclasses, cholesterol in medium HDL was inversely related to BW. An inverse association between HDL cholesterol and BW has been previously described in obese subjects (Misra *et al.*, 2011) and in insulin-treated GDM patients (Barrett *et al.*, 2013a). In our data, there was no significant interaction between metformin or insulin treatment in the association between medium HDL cholesterol and BW.

Total FA, SFA and MUFA were associated positively with BW, but these associations were attenuated at 36 gw. LA, total omega-6 FA and PUFA were related to higher BW, but this association was significant only in the metformin group and not in the combined analysis or the insulin group alone. Previously, maternal omega-3 FA have been positively and ARA but not total omega-6 FA inversely related to BW in population cohorts (Van Eijsden *et al.*, 2008; Grootendorst-van Mil *et al.*, 2018). Our results seem to be in contrast to these findings, although the regulation of fetal growth may be different in pregnancies complicated by GDM compared to general pregnant population.

There are only few studies focusing on how different GDM treatments affect the associations between maternal lipids and fetal growth. Most of the previous studies have included only or mostly women with GDM not on pharmacological treatment (Knopp *et al.*, 1992; Schaefer-Graf *et al.*, 2008, 2011). In a secondary analysis of the MiG trial, maternal HDL cholesterol at 36 gw correlated inversely with BW only among insulin-treated patients (Barrett *et al.*, 2013a). In our data, such an association was not found, which may be explained by higher pBMI and higher GWG in the MiG trial (Barrett *et al.*, 2013a), as the inverse relation between HDL cholesterol and BW is modified by maternal obesity (Misra *et al.*, 2011). Barrett *et al.* did not study the lipids that had different associations to BW depending on treatment in our study, i.e., cholesterol in VLDL subfractions, remnant cholesterol, TG in large lipoprotein particles apoB to apoA-1 ratio, apoB, LA, omega-6 FA, PUFA and total FA (Barrett *et al.*, 2013a).

We hypothesize that insulin could affect lipid metabolism and transfer in the placenta. This could explain the different associations between maternal lipids and BW between the insulin and metformin groups. Accordingly, we showed that the women whose VLDL cholesterol or apoB to apoA-1 ratio was in the highest quartile at the time of GDM diagnosis delivered heavier babies if assigned to metformin rather than insulin. The difference was not very marked and these findings need to be confirmed in a larger study. There were no differences between the whole treatment groups in terms of absolute or adjusted BW or incidences of LGA or SGA. Thus, while insulin may be more beneficial for mothers with hyperlipidemia, insulin therapy may have some drawbacks in terms of greater variability in glucose values. Indeed, the variation in glycemic control, as well as temporary hyperglycemias are associated with fetal growth (Law *et al.*, 2015).

Due to the large amount of intercorrelated lipid variables, we also ran PLS and PLS-DA analyses to study the associations between the maternal lipidome and BW. At baseline, the lipidome explained only 4.66% of the variation in BW (Q^2-value), based on a linear multivariate PLS model. At 36 gw, the predictive capability was even lower: 1.48%. This was less than anticipated and underlines the importance of other factors than the maternal fasting lipidome as determinants of fetal growth in well controlled GDM patients.

6.6.2 Cord serum metabolome

Amino acids, glucose, lactate and ketones

Transfer of several amino acids is altered in GDM pregnancies (Cetin *et al.*, 2005), but based on our data only histidine in cord serum seems to be related to BW. Previous studies have reported that histidine, glycine and taurine are decreased in SGA fetuses (Cetin *et al.*, 1990) and a population cohort study that histidine is positively related to BW (Hellmuth *et al.*, 2017c), albeit this association did not reach the Bonferroni-corrected p-value threshold. In our data, the relationship between cord serum histidine and BW was independent of treatment group and robust against adjustment for pBMI, GWG and maternal HbA1c. The reason why only histidine, of all amino acids, was associated with BW may be related to the close relationship between histidine and nucleotide metabolic pathways.

Two ketones, 3-hydroxybutyrate and acetone, were positively related to BW. Fetal production of ketones is minimal and 3-hydroxybutyrate in the cord serum is mostly of maternal origin, as 3-hydroxybutyrate crosses the placenta (Herrera *et al.*, 2006). Maternal ketones, including 3-hydroxybutyrate are increased in GDM (Montelongo *et al.*, 1992; Pappa *et al.*, 2007; Scholtens *et al.*, 2014; Dudzik *et al.*, 2017; Mokkala *et al.*, 2020b) and previous studies have shown that 3-

hydroxybutyrate and its metabolite acylcarnitine C4-OH are positively related to BW (Kadakia *et al.*, 2019b). The degree to which the fetus can utilize ketones for lipogenesis is not known (Herrera *et al.*, 2006).

Lipids

Inverse relationships between cord serum total TG, VLDL TG and BW were observed. These associations were stronger when adjusted for maternal pBMI, but did not reach statistical significance. Instead, there were significant inverse associations between the ratio of TG to phosphoglycerides, mean VLDL diameter and BW. Previously, cord blood TG has been inversely related to BW in pregnancies complicated by GDM (Schaefer-Graf *et al.*, 2008) or obesity (Patel *et al.*, 2018). It has been proposed that since larger fetuses have greater amounts of adipose tissue, TG uptake increases. Fetal hyperinsulinemia associated with GDM could also promote TG uptake in fetal peripheral tissues. In our study this association was most evident in TG-rich VLDL lipoproteins which transfer lipids into peripheral tissues. In FGR (Sanz-Cortés *et al.*, 2013; Miranda *et al.*, 2018) and SGA neonates (Nagano *et al.*, 2013) VLDL particle concentrations and VLDL TG are increased, possibly due to compromised uptake and utilization.

Cholesterol in very large and large HDL particles and the average diameter of HDL particles were positively related to BW, but total cholesterol was not. While the concentrations of LDL and VLDL cholesterol in cord serum are low, the HDL cholesterol is closer to the concentration in adult serum. The apolipoprotein composition of fetal HDL with a relative abundance of apolipoprotein E is, however, different from the composition in adults (Nagasaka *et al.*, 2002). HDL could thus be an important lipoprotein for facilitating fetal lipid transfer, since apolipoprotein E acts as a ligand for a variety of receptors, including LDL receptors. In our data, the associations between HDL diameter, HDL cholesterol and BW were significant after adjustment for pBMI, GWG or HbA1c. This suggests that fetal HDL transport may be an important regulator of fetal growth, partly independently of maternal glycemia. However, since all pregnancies in our study population were complicated by GDM the data is not representative of normoglycemic pregnancies. Cord serum HDL cholesterol has previously been positively related to IGF-1 (Nagano *et al.*, 2013) and negatively to SGA (Pecks *et al.*, 2012; Nagano *et al.*, 2013), further supporting the role of HDL cholesterol in fetal growth.

The long-chain PUFA are essential for fetal development and their accumulation in the fetus is enhanced in late pregnancy (Kuipers *et al.*, 2012). It has been proposed that the third trimester placenta has selectivity towards long-chain PUFA (Crawford *et al.*, 1976; Ortega-Senovilla *et al.*, 2009; Gil-Sánchez *et al.*, 2010), although recent findings do not support this mechanism (Ortega-Senovilla *et al.*, 2020).

In our study, a clear pattern of positive association between omega-6 FA and an inverse association between omega-3 FA and BW was observed. The PUFA to MUFA, or to total FA ratio was positively related to BW, while DHA and total omega-3 FA had inverse associations. This is partly in disagreement with previous studies, where most omega-3 FA, omega-6 FA and PUFA have been inversely and MUFA positively related to BW (Rump *et al.*, 2001; Hellmuth *et al.*, 2017c; Robinson *et al.*, 2018). These differences could be due to differences in study population or methodology, as none of these studies included exclusively pregnancies complicated by GDM, as was the case in our study. Moreover, the association may vary depending on which lipid component (TG, phospholipids or cholesterol esters) was studied (Elias *et al.*, 2001).

ARA may increase adiposity by promoting differentiation from preadipocytes into adipocytes (Gaillard *et al.*, 1989), while DHA has opposing effects (Kim *et al.*, 2006). This might explain the associations we found in pregnancies complicated by GDM. Also, a high omega-6 to omega-3 ratio in maternal and cord plasma is associated with infant obesity (Donahue *et al.*, 2011). Maternal serum LA and omega-6 FA were positively predictive for BW in the metformin, but not in the insulin group. In the associations between cord serum lipids and BW there were, however, no clear differences between the treatment groups. Only the association between the cord serum omega-6 to omega-3 ratio and BW was marginally stronger in the insulin (0.34; CI: 0.12, 0.56 SD/SD) compared to the metformin (0.22; CI: -0.010, 0.45 SD/SD) and the diet groups (0.045; CI: -0.12, 0.21 SD/SD).

In summary, cord serum FA are related to BW independent of maternal traits such as pBMI, GWG or HbA1c. Moreover, there was a positive association between omega-6 FA and BW in cord serum similar to maternal serum, suggesting that reducing maternal dietary omega-6 to omega-3 FA ratio could result in a decreased risk of LGA.

6.7 Associations between the maternal metabolome, inflammatory markers, IGFBP-1 and clinical outcomes

Overall, the associations between, on the one hand, maternal serum metabolites, inflammatory markers and IGFBP-1 phosphoisoforms and, on the other hand, perinatal outcome variables were weak. The incidence of many perinatal outcomes was low and the original randomized trial was powered to prove non-inferiority of the metformin compared to insulin treatment for BW alone (Tertti *et al.*, 2013). In that sense, this section should be considered more of an exploratory analysis.

6.7.1 Amino acids and glucose

The associations between baseline maternal serum amino acids, glucose, lactate and perinatal outcome variables were examined in the whole study population (diet, metformin and insulin-treated patients combined) and at 36 gw in the patients requiring pharmacological treatment (metformin and insulin groups). At baseline, several amino acids and glucose were inversely related to gestation length at delivery. The association was strongest for alanine and tyrosine, but also all BCAA had an inverse association with gestation length, although not statistically significant after FDR-correction.

This association between amino acids and gestation length could be explained by maternal insulin resistance, since maternal amino acids are inversely related to insulin sensitivity in pregnancy (Sandler *et al.*, 2017; Liu *et al.*, 2020). Another ominous sign of insulin resistance, elevated fasting glucose, has been linked to a risk of preterm birth and to overall decreased length of gestation (Magnussen *et al.*, 2011). Furthermore, mothers with GDM and high insulin resistance, compared to mothers with GDM characterized predominantly by impaired insulin secretion rather than insulin resistance, are at increased risk of several adverse pregnancy outcomes in addition to reduced duration of gestation (Powe *et al.*, 2016; Benhalima *et al.*, 2019; Immanuel *et al.*, 2020). Although the rate of induction of labor was recorded in our study, the indications were not. Hence, it is not possible to assess how maternal factors, such as suboptimal glycemic control or hypertensive complications, or fetal factors such as impaired or excessive growth or a nonreassuring fetal status, may have contributed to this association.

Glutamine was marginally higher at baseline in the patients who did not require pharmacological treatment compared to the metformin and insulin groups combined. Also, baseline glutamine was related inversely to GWG in the whole population, and glutamine at 36 gw was associated positively with hypertensive disorders of pregnancy in the combined pharmacological treatment group. The differences in associations between baseline and 36 gw measures may be partly due to a lack of metabolomic data from the diet group at 36 gw but also to development of maternal hypertension and initiation of antihypertensive medication by 36 gw.

Reduced, rather than increased, glutamine concentrations have been previously reported in preeclampsia (Hsu *et al.*, 2005; Dunn *et al.*, 2009). These results are in conflict to ours and may be related to heterogeneous condition of preeclampsia (Benton *et al.*, 2018). Of course, our population of GDM may differ from the previous studies, which have been small.

Not surprisingly, glucose at baseline was a significant predictor of several adverse outcomes. The association was, however, attenuated at 36 gw, possibly due to successful antihyperglycemic treatment.

6.7.2 Lipids

Maternal lipids have previously shown to be related to a risk of preeclampsia (Kenny *et al.*, 2010). We did not, however, find a significant association between the maternal lipidome and hypertensive disorders. This finding may be explained by the low rate of hypertensive complications in our study population, which reduces the statistical power of our study in this respect.

Instead, maternal lipids were related to total GWG, although most of these associations were attenuated when adjusted for pBMI. The association between maternal serum lipids and pBMI is well known (Hellmuth *et al.*, 2017b; Sandler *et al.*, 2017). The MUFA to total FA ratio at baseline was inversely related to GWG. At 36 gw TG in large HDL and mean diameter of HDL particles, which probably also reflects the increase in HDL TG content, were positively related to GWG. The only significant association after adjustment for pBMI was between the SFA to total FA ratio and GWG. The associations between maternal lipids and GWG are reportedly minimal also in non-GDM pregnancies (Hellmuth *et al.*, 2017b; Lindsay *et al.*, 2018). Maternal lipid profile does thus not seem to be an important predictor of GWG.

6.7.3 Inflammatory markers and IGFBP-1 phosphoisoforms

The predictive value of maternal inflammatory markers and IGFBP-1 phosphoisoforms with regard to adverse pregnancy outcomes was evaluated at baseline and at 36 gw among the patients who needed pharmacological treatment. At both time points non-pIGFBP-1 was inversely related to GWG. Besides the association between IGFBP-1 and GWG, IGFBP-1 was also inversely related to a more favorable metabolic profile at baseline: lower pBMI and lower C-peptide, respectively. Thus, we would have expected a higher IGFBP-1 concentration to predict a lower BW in line with previous studies (Jansson *et al.*, 2008; Åsvold *et al.*, 2011; Lappas, 2015), but none of the studies were focused in GDM and in one study (Lappas, 2015) the women with GDM were excluded. Moreover, the previous studies did not assess different phosphoisoforms nor did we find a significant association between the most abundant highly phosphorylated isoform and BW.

Although not statistically significant after the Bonferroni correction, low-pIGFBP-1 at baseline was inversely related to the risk of induction of labor, and high-pIGFBP-1 at 36 was inversely related to the risk of cesarean delivery. These associations were insignificant when adjusted for pBMI, suggesting that IGFBP-1 phosphoisoforms reflect the overall metabolic health of the subjects. The inverse association between low-pIGFBP-1 and induction of labor was stronger in the metformin group than in the insulin group and, taking into account that the rate of

induction was higher in the insulin group, this may reflect different indications for induction of delivery between the groups, although these data were not recorded.

Baseline MMP-8 correlated positively with pBMI and its concentration did not change during pregnancy. At 36 gw it was weakly associated with lower BW, independent of pBMI. Previously MMP-8 has been associated with chorioamnionitis (Kim *et al.*, 2015) and preterm delivery (Ashford *et al.*, 2018) and this weak inverse association with BW in our study could reflect chronic placental inflammation, although more studies on the subject are needed.

6.8 Clinical implications and future prospects

In GDM, insulin treatment is more effective in ameliorating dyslipidemia, including high total TG, VLDL TG, VLDL cholesterol and HDL TG, than metformin treatment. Furthermore, the associations between maternal lipids and BW were stronger in the metformin group. These findings have several possible implications. First, the short duration of a worse lipid profile in the third trimester normalizes fast postpartum (Barrett *et al.*, 2013a) and is therefore unlikely to cause adverse consequences on maternal cardiovascular health. Follow-up studies on the cardiovascular health are still warranted. Second, the relative increase in maternal lipids in the metformin group did not result in higher BW. The associations between maternal lipids and BW were, however, stronger in the metformin group and, as demonstrated, the fraction of patients who had highest VLDL cholesterol or apoB to apoA-1 ratio delivered heavier babies if treated with metformin instead of insulin. Future studies should focus on characterizing the patients for whom metformin treatment is more beneficial than insulin and vice versa. Modifying the diet regimen of patients on metformin by increasing low glycemic index carbohydrates and reducing dietary fat (Hernandez *et al.*, 2018) might lead to improved pregnancy outcomes, and this assumption merits further study.

Metabolomics and maternal serum inflammatory markers might be useful to predict the need for pharmacological therapy or adverse neonatal outcomes in GDM. In this study, we did not succeed in identifying suitable biomarkers, in single or combination, for this end. However, with a different approach to the maternal metabolome, e.g., measuring the metabolome after a glucose load or using MS based techniques, could yield different results. Nevertheless, our results demonstrate that the associations between biomarkers and perinatal outcomes may vary by maternal treatment. Classifying GDM patients by defects predominantly in insulin secretion or insulin resistance reveals different risk profiles (Powe *et al.*, 2016; Benhalima *et al.*, 2019; Immanuel *et al.*, 2020). An analogous stratification by the maternal metabolome might help to individualize the treatment of GDM.

Metformin exposure in utero has been shown to relate to increased offspring weight in childhood (van Weelden *et al.*, 2018; Hanem *et al.*, 2019). This association could be reflected in the cord serum metabolome. We showed, however, that maternal treatment of GDM has no impact on neonatal cord serum lipids, ketones or lactate. Cord serum alanine was increased in the metformin group when compared to insulin and diet groups. Neonatal alanine was not associated with BW, but the mechanism by which alanine increases under the influence of metformin and the possible long-term implications of this finding require further studies.

7 Summary/Conclusions

This thesis presents a comprehensive metabolic profiling of GDM patients treated with metformin, insulin or diet and lifestyle modifications only. The total study population was rather large (n > 300) and, in addition to maternal sera, neonatal cord serum samples were analyzed. The effects of metformin on the maternal metabolome and inflammatory markers were characterized and the associations between these biomarkers, BW and clinical outcome variables were studied. The following conclusions can be made:

1. There were no major differences between maternal serum metabolites, inflammatory markers or IGFBP-1 phosphoisoforms between patients requiring and not requiring pharmacological treatment, and therefore maternal metabolome is scarcely useful for identifying patients who will require pharmacological treatment. (Studies I–III)
2. In comparison to insulin, metformin treatment of GDM has distinct effects on maternal metabolism and, to some extent, also on inflammatory markers and IGFBP-1 phosphoisoforms. The concentration of serum alanine, total TG, VLDL TG, total FA, GlycA and non-pIGFBP-1 increased more in the metformin-treated patients than the insulin-treated patients. (Studies I–III)
3. Maternal isoleucine, total and the VLDL TG, VLDL cholesterol and apoB to apoA-1 ratio at the time of GDM diagnosis were positively related to BW. The association between lipids and BW was stronger in the metformin group than the insulin group. Mothers with high VLDL cholesterol or a high apoB to apoA-1 ratio at the time of GDM diagnosis may benefit from insulin rather than metformin treatment with regard to a lower BW. There were no significant associations between maternal inflammatory markers, IGFBP-1 phosphoisoforms and BW. (Studies I–III)
4. It was reassuring to find that treatment of GDM has only minimal effects on the cord serum metabolome. The only exception was a clear increase in alanine in the metformin group. The study design did not allow

differentiation between a direct effect of metformin on the fetal metabolism and an indirect effect on maternal or placental metabolism. Cord serum lipids, including VLDL TG, cholesterol in very large HDL, PUFA, omega-3 and omega-6 FA, are related to BW. The fetal supply and metabolism of these metabolites could be important determinants of fetal growth in utero. (Study IV)

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