Placental and humoral alterations in gestational diabetes

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät

der Eberhard Karls Universität Tübingen

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

vorgelegt von

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Tübingen

2018

Gedruckt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Eberhard Karls Universität Tübingen.

Tag der mündlichen Qualifikation:	29.05.2018
Dekan:	Prof. Dr. Wolfgang Rosenstiel
1. Berichterstatter:	Prof. Dr. Harald Staiger
2. Berichterstatterin:	Prof. Dr. Gisela Drews

Die Zeit

So wandelt sie, im ewig gleichen Kreise,
Die Zeit nach ihrer alten Weise,
Auf ihrem Wege taub und blind,
Das unbefangene Menschenkind
Erwartet stets vom nächsten Augenblick
Ein unverhofftes seltsam neues Glück.
Die Sonne geht und kehret wieder,
Kommt Mond und sinkt die Nacht hernieder,
Die Stunden die Wochen abwärts leiten,
Die Wochen bringen die Jahreszeiten.
Von außen nichts sich je erneut,
In dir trägst du die wechselnde Zeit,
In die nur Glück und Begebenheit

Ludwig Tieck

List of contents

List of abbreviations

APJ	Apelin receptor
APL	Apelin
BMI	Body mass index
CE	Cholesterol ester
DG	Diglycerides/diacylglycerols
DNA	Deoxyribonucleic acid
FABP	Fatty acid binding protein
FAT	Fatty acid translocase
FC	Fold change
FFAR	Free fatty acid receptor
FGF	Fibroblast growth factor
FTO	Fat mass and obesity-associated protein
G-CSF	Granulocytecolony stimulating factor
GCT	Glucose challenge test
GDM	Gestational diabetes mellitus
GM-CSF	Granulocyte macrophage colony-stimulating factor
HAPO	Hyperglycemia and Adverse Pregnancy Outcome
hCG	Human chorionic gonadotropin
hsCRP	High-sensitivity C-reactive protein
IADPSG	International Association of the Diabetes and Pregnancy Study Groups
IGT	Impaired glucose tolerance
IGFBP2	Insulin like growth factor binding protein 2
IFN	Interferon
IL	Interleukin
ISI	Insulin sensitivity index
IR	Insulin resistance
LIPG	Endothelial lipase
LPL	Lipoprotein lipase
Mio	Million
miRNA	MicroRNA
MIP	Macrophage inflammatory proteins
MUFA	Mono-unsaturated fatty acids
NCD	Non-communicable diseases
NEFA	Non-esterified fatty acids
NF_KB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGT	Normal glucose tolerant
NZO	New Zealand obese
OGTT	Oral glucose tolerance test
PEACHES	Programming of Enhanced Adiposity Risk in childhood-Early Screening
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-α
piRNA	PIWI-interacting RNAs
PL	Phospholipids
PLIN	Perilipin
PPAR	Peroxisome proliferator-activated receptor
PUFA	Poly-unsaturated fatty acids
qPCR	Quantitative polymerase chain reaction
RANTES	Regulated on activation
RNA	Ribonucleic acid
RT	Room temperature
SFA	Saturated fatty acids
SLC27A1, 4	Solute Carrier Family 27 Member 1, 4
T1D	Type-1 diabetes

T2D	Type-2 diabetes
TCF7L2	Transcription factor 7-like 2
TG	Triglycerides/triacylglycerols
TLR	Toll like receptor
TNF-α	Tumor necrosis factor
tRNA	Transfer RNA
VLDL	Very-low-density lipoproteins
VEGF	Vascular endothelial growth factor
WBC	Whole blood cells

Introduction

1. *Growing incidence of diabetes*

The burden of non-communicable diseases (NCD) is a major factor contributing to world-wide deaths. In low- and middle-income countries, NCD count up to 80% of all annual deaths. Therefore, NCD have a major impact on social and economic development. At the individual level, NCD are associated with reduced income, increased risk of poverty and risk of early death (WHO, 2014). Reduced income diminishes life quality and limits the access to prevention and therapy. Currently, in industrialized economies, a big financial effort is conducted to identify and treat NCD and NCD-associated complications. A steady increase of prevention programs – both at the individual and policy-making level - is observed. The burden of NCD includes diabetes next to cancer, chronic respiratory diseases, and cardiovascular disease. In 2015, 415 million people were estimated to suffer from diabetes [2]. By 2040, a 50% increase of the world-wide diabetes (mainly type-2 diabetes (T2D)) incidence rate is expected. Major increases are expected in the fast-changing and fast-growing societies of low- and middle-income countries, such as Africa, Latin America and South-East Asia [2]. Figure 1 summarizes the estimated number of people with diabetes world-wide in 2015 versus 2040.

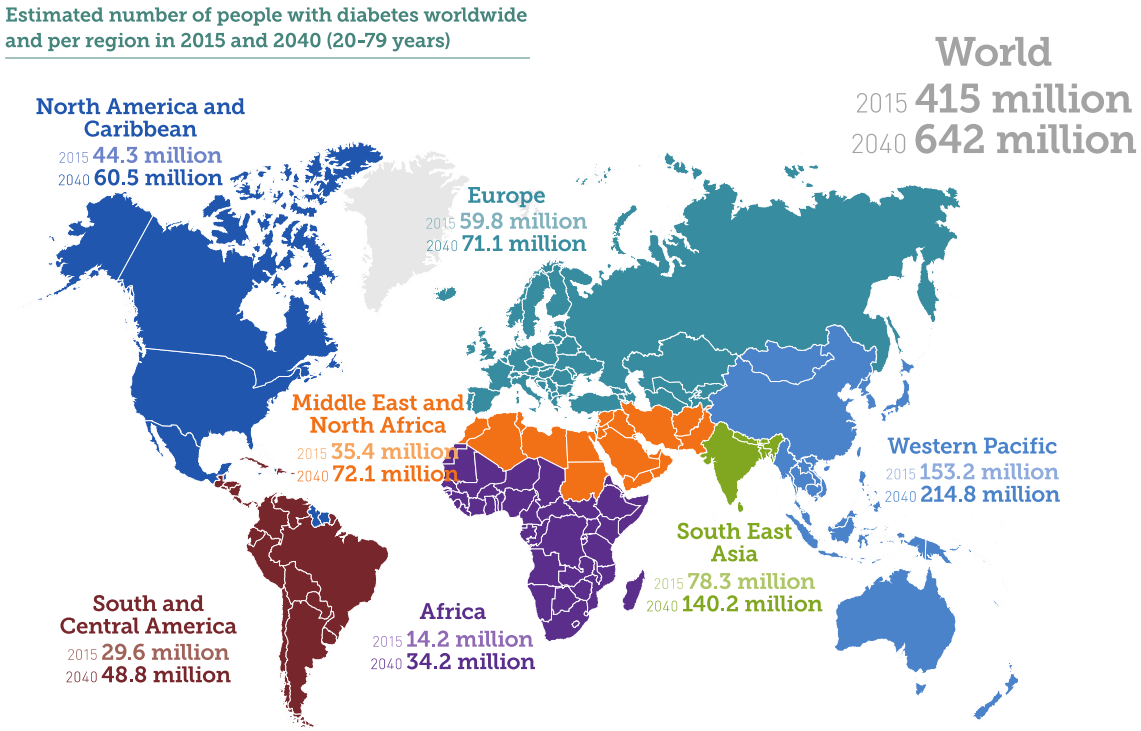

Figure 1: Number of people with diabetes worldwide and per region in 2015 and 2040 (20-79 years).
Source: IDF atlas [2]

The world-wide elevated prevalence of overweight and obesity is the main risk factor for the development of T2D. In the modern days, adverse effects of elevated weight can already be found in young children (0-5 years). In 1990, 32 Mio young children were overweight and obese. Currently, 41 Mio and in 2025 over 70 Mio young children will have elevated weights [4]. These epidemiological data propose that offspring's early metabolic health is associated with T2D incidence rates later in life. Causes of elevated children's weight may be related to parental health before conception and maternal health during pregnancy [5, 6].
The whole phenomenon of fast growing T2D incidence rates is described by the term "transgenerational diabetes" [2]. Causes contributing to transgenerational diabetes need to be investigated. Identification of prevention strategies may interrupt the transmission of adverse parental metabolic health status to new generations.

2. Transition from "thrifty genes" to "fetal programming" as contributors to transgenerational diabetes

In the 1960s James Neel published several articles describing the hypothesis of the "thrifty genes". Neel explained why (sub-)populations are more prone to develop obesity and T2D in the modern days [7]. According to the "thrifty gene hypothesis", evolution selected people with a genotype enabling them to survive periods of feast and famine. The major advantage of this genotype is the capacity to store fat effectively. Today, in our modern societies, this survival advantage has become a big disadvantage for the human population. It is associated with the burden of obesity [8] and T2D. Indeed, gene variants were identified in defined sub-populations (such as Pima Indians) [9, 10] that are the main cause for high prevalence of T2D, there [11]. Various common variants in genes, such as transcription factor 7-like 2 (*TCF7L2)* or fat mass and obesity-associated protein (*FTO)*, were found associated with an elevated risk for T2D also in western societies [12, 13]. However, only modest support from population-wide genetic analysis has been found explaining the worldwide increase of incidence of obesity and T2D.

Epidemiological reports of Barker and Hales [14-16] describe associations of maternal nutritional status and fetal risk for developing metabolic diseases. The hypothesis of the "thrifty phenotype" or "fetal programming" proposed by these authors describes that adaptation in the *in-utero* period contributes to the increased risk of T2D in the offspring of mothers with malnutrition. Ravelli et al described 1978 that men born in and after the Dutch hunger winter had higher rates of obesity when the period of nutritional deprivation was in early pregnancy and reduced rates of obesity in late fetal and early postnatal caloric restriction [17]. These findings gave rise to the suggestion that both, maternal over- and malnutrition could modify fetal risk to develop obesity and T2D later in life.

In parallel to these epidemiological studies, the hyperglycemia-hyperinsulinemia hypothesis [18] was developed by Pedersen, based on findings mainly in type-1 diabetic (T1D) women. He proposed that “maternal hyperglycemia results in fetal hyperglycemia and, hence, in hypertrophy of fetal islets tissue with insulin-hypersecretion. This again means a greater fetal utilization of glucose. This phenomenon will explain several abnormal structure and changes found in the newborn” [18]. The elevated maternal glucose levels can pass the placenta in a concentration-dependent manner [19] and lead to increased fetal insulin secretion upon the tenth week of pregnancy [20]. Fetal insulin itself is a major *in-utero* growth factor [21]. Compared to T1D, gestational diabetes (GDM) is usually associated with elevated maternal weight and other metabolic factors, such as elevated maternal plasma lipids [22, 23]. These metabolic characteristics may influence the placenta and fetal metabolism. In response to this discussion, the concept of “fuel-mediated teratogenesis” was established by Freinkel in the 1980s [24, 25]. His extension of the hyperglycemia-hyperinsulinemia hypothesis was supported by the finding that macrosomic offspring exists also in well controlled GDM pregnancies. [26]. Freinkel’s proposal is still accepted and under current investigation.

The “Hyperglycemia and Adverse Pregnancy Outcome (HAPO) Study”, a five-year prospective observational study investigated the impact of maternal health during pregnancy on fetal development [27]. In the HAPO study, 25,000 women in ten countries were monitored during pregnancy. Central laboratory measurements of maternal and fetal blood during and after birth, socioeconomic and maternal health history, as well as neonatal anthropometrics and data from 75-g oral glucose tolerance tests (OGTT) were collected. Early results of the HAPO study [27] and similar smaller studies indicate that both, maternal obesity and elevated maternal glucose levels (also below GDM diagnostic criteria) are strong and independent factors associated with adverse pregnancy outcome such as elevated birth weight, elevated amount of body fat or fetal hyperinsulinism. Both are shown to affect fetal risk to develop obesity and T2D later in life [28-31].

3. Maternal health during pregnancy contributes to fetal health

The observational findings of the HAPO study led to further studies trying to understand pathomechanisms induced by maternal obesity and GDM. In order to understand the relevance of adverse maternal health for fetal health, the physiology of normoglycaemic pregnancies and the role of the placenta will be explained, first.

Physiological regulation of metabolism in normoglycaemic pregnancies

The first two trimesters of pregnancy are characterized by an increase of maternal adipose tissue mass due to elevated caloric intake [32]. Additionally, insulin resistance (IR) develops during pregnancy. Pregnancy hormones (lactogen, estrogen, progesterone, cortisol) [33-35] and circulating factors known to be elevated during pregnancy, such as adiponectin, tumor

necrosis factor (TNF-α), leptin, and high-sensitivity C-reactive protein (hsCRP), are discussed to be relevant for the development of IR in all pregnancies (not only pregnancies accompanied by GDM) [36, 37]. The pancreas function can compensate the growing demand of insulin by (potentially lactogen-induced) adaptive β-cell mass expansion and increase of insulin secretion [38, 39]. In the third trimester, elevated IR and increasing fetal fuel demand lead to loss of maternal adipose tissue mass. This is associated with an increase in maternal plasma very-low-density lipoproteins (VLDL) and free non-esterified fatty acids (NEFA) [40] leading to a further reduction of insulin sensitivity in late pregnancy [34]. The uptake of maternal lipids by the placenta and finally the transport to the fetal circulation are well controlled [3, 19]. Maternal lipoproteins are degraded extracellularly (endothelial lipase (LIPG) and lipoprotein lipase (LPL)). NEFA are taken up by fatty acid transport proteins (SLC27A1 and SLC27A4), fatty acid translocase (FAT) as well as the plasma membrane fatty acid binding protein (p-FABP) [41]. Within the syncytiotrophoblast, NEFA are bound to FABP3 [42] and enter metabolic paths such as beta-oxidation or have their relevance in fatty acid signaling. They may also be reesterified to phospholipids (PL) and triglycerides (TG) which are stored within perilipin 2 (PLIN2)-positive lipid droplets in the syncytiotrophoblast [43]. To supply the growing fetus with fatty acids, the syncytiotrophoblast can release NEFA to the fetal circulation. Bound to transport proteins (α2-Heremans-Schmid glycoprotein alias fetuin A), they are taken up from the fetal circulation by the fetal liver where they are metabolized or esterified for further transport to fetal tissues [44].

Structure and function of the placenta

The placenta is an organ being in contact with the maternal and the fetal blood circulation. Via the placenta, circulating nutrients and oxygen of the maternal blood are delivered to the fetus, and fetal and placental products are dispensed to the mother. Additionally, the placenta is an endocrine organ regulating pregnancy and fetal development [45]. Placental adaptations in response to maternal metabolism may therefore influence fetal metabolism and contribute to *in-utero* programming.

Placental villi consist of three layers of components, as indicated in Figure 2. The outmost continuous layer consists of trophoblasts (undifferentiated cytotrophoblasts and differentiated syncytiotrophoblasts). These cells are in direct contact with maternal blood. Within the villous core are mesenchymal cells, fibroblasts and macrophages (Hofbauer cells). Finally, there are fetal endothelial cells which collect oxygen and nutrients and release them to the fetal venous cord blood [45].

The trophoblasts are the unique cell type of the placenta and therefore key components for maternal-fetal communication. They are the structural and biochemical barrier between mother and fetus [3, 45]. Moreover, they can store nutrients and are an endocrine source of placental hormones (i.e. estrogen, progesterone, human chorionic gonadotropin (hCG)).

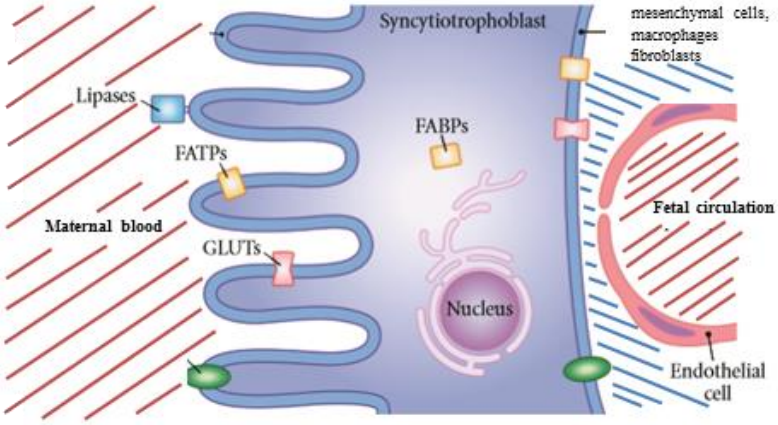

Figure 2: Functional layers of the placenta and proteins being involved in nutrient transport across the placenta.

For transfer of lipids, extracellular lipases release fatty acids from maternal lipoproteins and intracellular binding proteins (FABPs) guide the fatty acids within the cytosol of the syncytiotrophoblast. Source: Image adopted from [3]

Influence of maternal weight on fetal health

Epigenetic programming and metabolic adaptions to the environment of germ cell [46, 47], the placenta, and the developing fetus [48] are discussed to modify offspring's lifelong health [28]. During adaptive periods the environment can make imprints on the metabolism of a single individual [49].

Maternal overweight and obesity were shown to modify placental lipid metabolism and storage [50]. These modifications have central relevance for metabolic programming of the fetus in the *in-utero* period [51]. According to a German National Consumption Survey (Nationale Verzehrsstudie ll) [52], 21% of women between 30-39 years of age are overweight and 14% of the women are obese in Germany. In Mexico, an exemplary transition country, 65% of the women are overweight and 32% are obese [4]. Maternal obesity in early pregnancy was shown to double the risk of obesity in early childhood [53]. Furthermore, it was shown that maternal pre-pregnancy body-mass-index (BMI) can predict in young adults adiposity [54] and T2D [28].

Influence of maternal GDM on fetal health

Another independent factor for increased risk of the offspring to develop T2D is maternal GDM [29]. Following the diagnostic recommendation of the International Association of Diabetes and Pregnancy Study Groups (IADPSG) [55], GDM is defined as "any degree of glucose intolerance with onset or first recognition during pregnancy". Clinical diagnosis criteria of GDM based on a 75-g OGTT are the following: fasting glucose level: 92mg/dl (5.11mol/l), 1-h plasma glucose: 180mg/dl (10.0mol/l), 2-h plasma glucose: 153mg/dl (8.5mol/l). For the diagnosis of GDM, one or more of these values must be equaled or exceeded. On the base of these diagnostic criteria, 13% of all live birth pregnancies were accompanied by GDM in 2015 in Germany [56]. In contrast, in the more overweight and obese population of Mexico, the prevalence was around 30% in 2012 [57]. Literature proposes that adverse GDM-associated effects on fetal future health are more severe compared to BMI effects. Epidemiological data of small GDM follow-up cohorts show that

offspring of diet-treated GDM-women have an odds-ratio of 7.76 to develop T2D later in life. This is reflected by a T2D incidence rate of 21% till the age of 22 years [5, 58].

Currently, the pathophysiology of GDM is not understood. However, GDM is clinically characterized by elevated glucose, insulin and plasma NEFA values as well as a degree of IR that is no longer compensated for by insulin hypersecretion.

Impact of GDM-associated metabolic derangement on fetal future health

Metabolic derangement contributes to both, the progression of maternal IR and elevated fetal risk for obesity and T2D:

- Increased plasma NEFA arising from insulin-resistant hypertrophic adipose tissue were shown to associate with lipoinflammation and the development of peripheral (skeletal muscle cells [59], hepatocytes [60], adipocytes [61], and pancreatic islets [62, 63]) and central [64, 65] IR. Additionally, maternal lipids are a candidate to explain fetal macrosomia independently of maternal glucose levels in GDM [26, 66]. They were proposed to affect placental and fetal lipid metabolism [25, 67] and mediate *in utero* the offspring's risk the develop T2D later in life [26, 66].
- Increased maternal glucose levels, also resulting from maternal IR, can pass the placenta in a concentration-dependent manner [29, 68]. In T1D, elevated fetal glucose concentrations lead to fetal hyperinsulinemia [69, 70]. For the lower glucose concentrations in GDM, this remains to be shown. Finally, elevated fetal insulin levels may have an impact on the development of IR *in utero* [71-73].

The metabolic derangement in GDM may result from elevated maternal weight or weight gain during pregnancy. However, this is not the only cause for development of GDM, as not all GDM women have an elevated BMI or weight gain during pregnancy [66]. The pathogenesis is discussed to be accompanied by reduced pancreatic adaptation to increased insulin demand [74] and elevation of various placental hormones [35]. For instance, placental lactogen contributes to β-cell mass adaptation during pregnancy [39], lipolysis of adipose tissue [75] and was shown to cause peripheral IR [76]. Apelin (*APL*), another hormone being also released by the placenta is discussed to have a regulatory function in energy metabolism. In more details, this hormone was shown to enhance glucose uptake [77], lipolysis and fatty acid oxidation [78], peripherally. Additionally, central actions of *APL* were proposed to contribute in the development of T2D (i.e. by acting on hypothalamus) [79-81]. GDM-associated expression changes of this placental hormone could therefore contribute to the progression of GDM and adverse fetal programming. In pregnant women [82] elevated plasma APL levels were found. In pregnancy, placental APL release was proposed to contribute to elevated maternal APL plasma levels [82]. Its role in fetal development and programming is currently under investigation. Recently, it was shown that maternal

overnutrition elevates and maternal undernutrition reduces maternal and fetal plasma APL levels [83, 84]. Furthermore, maternal GDM was shown to be associated with elevated APL plasma levels compared to NGT women [85]. Additionally to systemic effects, placental APL release may enhance placental vascular functions and angiogenesis by activation of the apelin receptor (*APJ*) [82, 86].

Impact of GDM-associated epigenetic changes on fetal future health

Furthermore, GDM was shown to influence deoxyribonucleic acid (DNA) methylation patterns of the placenta and fetus [87-89]. In details, results of the Dutch hunger winter study in adults [90] and recent studies on epigenetic influences of GDM [91] in young children indicate that DNA methylation differences in blood cells can be observed in response to maternal nutritional and health status during pregnancy. The findings in adults indicate that epigenetic fetal programming may have effects on the whole lifetime of an individual. Additionally to DNA methylation differences, differential expression of microRNA (miRNA) in response to GDM-associated conditions [92-95] could contribute to epigenetic fetal programming [96].

In general, expression of cell-type-specific genes is regulated epigenetically. Therefore, findings in easily accessible cells (i.e. blood cells) may not necessarily reflect epigenetic differences present in tissues relevant for disease progression [97]. However, our study [98] of Insulin Like Growth Factor Binding Protein 2 (*IGFBP2*) DNA methylation (a protein involved in the regulation of insulin sensitivity [99]) indicated that certain epigenetic alterations can be similar in different cell types under similar metabolic conditions (such stable alterations are known as metastable epigenetic marks). As shown in Figure 3, DNA methylation of the *IGFBP2* gene was significantly elevated in murine liver of New Zealand obese (NZO) mice and in whole blood cells (WBC) of men with impaired glucose tolerance (IGT).

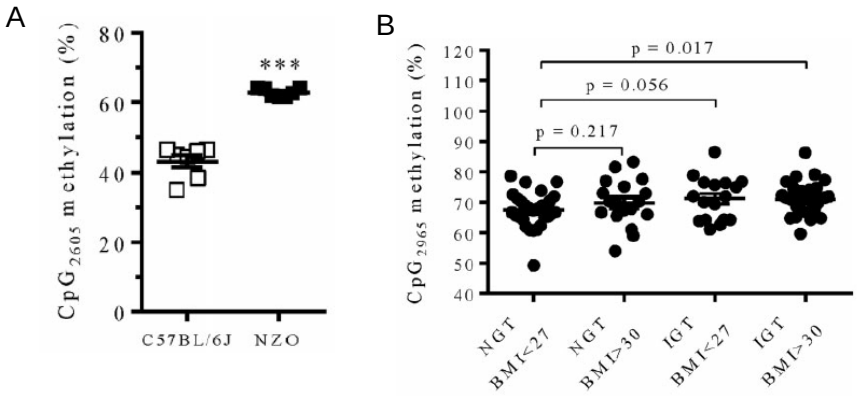

Figure 3: Comparison of IGFBP2 DNA methylation in murine liver and human whole blood cells. A: DNA methylation of CpG $_{2605}$ in murine liver of C57BL/6J and NZO mice. Both strains are prone to develop severe obesity, but only NZO mice are highly susceptible to diabetes. All data represented as mean ± SEM of N=21animals per group, respectively. B: DNA methylation of CpG$_{2965}$ in whole blood cells from human subjects classified into normal glucose tolerant with BMI<27 (NGT.BMI<27) or BMI>30 (NGT.BMI>30) or impaired glucose tolerant with BMI<27 (IGT.BMI<27) or BMI>30 (IGT.BMI>30). ***p<0.001 compared with untreated control group [98].

Based on these marks, epigenetic differences in easily accessible compartments, such as whole blood, have the potential to reflect epigenetic differences in maternal and fetal tissues relevant for GDM progression or fetal programming [95, 97].

Scientific goal

Elevated maternal pre-pregnancy BMI was shown to associate with elevated placental lipid content, expression of enzymes and transporters involved in lipid metabolism. Moreover, elevated expression and activity of inflammatory pathways were seen in the placenta of overweight and obese women [100]. According to results of long-term follow up studies, BMI and GDM are independent contributors to adverse fetal programming [68]. The epidemiological findings propose that GDM has stronger adverse influences on the offspring's programming [5, 6]. Recent findings indicate that maternal GDM, but not maternal BMI, is associated with *in-utero* development of fetal IR [30, 71, 101]. However, the underlying mechanisms are currently not understood.

Therefore, one goal of this work was to investigate effects of GDM, independently of BMI, on maternal plasma and placental lipid profiles. Furthermore, the effects of prominent GDM-associated lipid species on placental gene expression were investigated. Of special interest were genes involved in lipoinflammation, as earlier studies showed NEFA-mediated inflammatory response to be relevant for development of IR. Of further interest were placental hormones such as *APL* which was shown to be relevant in metabolic regulation. Hence, primary trophoblasts of normal glucose tolerant (NGT) and GDM women were stimulated with NEFA and expression changes of inflammatory genes, *APL*, and *APJ*, the APL receptor, were quantified. Biological relevance of the observed placental differences in inflammatory gene expression was investigated by comparison of placental macrophage contents in GDM and NGT women. Finally, to associate placental findings with fetal metabolism and the *in-utero* development of IR, cord blood lipid profiles, inflammatory patterns and APL concentrations were compared in offsprings of NGT and GDM women.

A further goal of this work was to investigate how GDM contributes to epigenetic differences [46, 98, 102]. Therefore, whole-blood miRNA (WBC, lymphocyte and plasma miRNA) expression was compared between NGT and GDM women. In order to characterize which GDM-associated metabolic stimuli could be relevant for blood cell miRNA expression differences, primary lymphocytes of NGT and GDM were stimulated with insulin and glucose. Finally, the relevance of *in-vitro* findings was tested by correlating maternal insulin levels with WBC miRNA expression patterns.

In conclusion, the overall scientific goal of this work was to investigate the BMI-independent influence of maternal GDM on placental lipoinflammation and whole blood miRNA pattern. Based on these goals, insights in fetal GDM-associated metabolism and the development of *in-utero* IR should be gained.

Results

Project I: BMI-independent effects of maternal GDM on placental lipoinflammation

The aim of this Project I was to investigate effects of GDM, independently of BMI, on maternal plasma and placental lipid profiles. As SFA-mediated activation of toll like receptor (TLR)-signaling was shown to contribute to the release of inflammatory markers [63, 103, 104], the influence of GDM on whole placenta and trophoblast-specific lipoinflammation was investigated. Proinflammatory cytokines such as *TNF-α* and interleukin 6 (*IL6*) were shown to associate with the development of IR in obese adults, earlier [59, 105, 106]. To approximate how lipid-associated alterations of the placenta could contribute to *in-utero* development of fetal IR, we compared a wide range of inflammatory parameters and NEFA in cord blood samples of NGT and GDM offspring.

Metabolic and anthropometric characterization of pregnant women

Table 1 shows clinical characteristics of each group of participants. Clinical characterization of the pregnancy cohort was conducted by the physicians and clinical scientists of the Clinical Study Center of the Department of Internal Medicine, Division of Endocrinology, Diabetology, Angiology, Nephrology and Clinical Chemistry, University Hospital Tübingen. Clinical characterization of the trophoblast donors and the Programming of Enhanced Adiposity Risk in CHildhood – Early Screening (PEACHES) cohort was conducted by the collaboration partners Gernot Desoye, Medical University Graz, and Regina Ensenauer, Ludwig Maximillians University Munich and Heinrich Heine University Düsseldorf, respectively. The OGTT or glucose challenge test (GCT) conducted in pregnancy week 27±2 (pregnancy cohort) or 22±1 (PEACHES), showed significantly increased levels of fasting, 1-h and 2-h glucose in the GDM women compared to the NGT group ($P<0.02$, all). No statistically significant group differences were found for maternal smoking behavior, mode of delivery or fetal sex ($P>0.05$, all). A flow chart indicating which analyses were conducted in which cohort is shown in supplemental Figure 1 (S.71).

Maternal plasma lipid analysis

In multiple linear regression analysis (confounding covariates: BMI, gestational age, fetal sex), total and saturated (SFA) NEFA concentrations were elevated in GDM compared to NGT women ($P\leq0.05$, Figure 4A, supplemental Figure 2A (S.71). Most abundant plasma NEFA were palmitate (C16:0), stearate (C18:0), oleate (C18:1-n9), and linoleate (C18:2-n6, Figure 4B). In plasma of GDM, women higher concentrations of palmitoleate (16:1-n7), stearate as well as docosahexaenoate (C22:6-n3) were found ($P\leq0.05$, Figure 4B, C). Furthermore, statistical trends for elevated amounts of palmitate, vaccenate (C18:1-n7), oleate, arachidonate (C20:4-n6), and nervonate (C24:1-n9) were present ($P\leq0.1$). Other lipid fractions were not analyzed within this project. Marketa Kovařova, Department of Internal Medicine, Division of Endocrinology, Diabetology, Angiology, Nephrology and Clinical

Chemistry, University Hospital Tübingen, conducted the quantification of maternal plasma lipids by gas chromatography.

Table 1: Anthropometric and metabolic characteristics of the participants From smaller subgroups of the pregnancy cohort, placental mRNA analysis, lipid profile analysis and protein quantification were conducted. From a subgroup of the PEACHES cohort, only 1-h glucose values of a 50-g glucose challenge test (GCT) were available (N=30). Mid-pregnancy was defined as gestational week 27±2 (pregnancy cohort) or 22±1 gestational weeks (PEACHES cohort). All data are given as absolute numbers or means ± SD. Group differences of numeric characteristics were calculated by two-tailed Student's t-test. Group differences of fetal sex, smoking behavior and mode of delivery were calculated using Chi-square test. P-values ≤0.05 were considered as statistically significant and are marked by using bold fonts. [a]Mid-pregnancy body weight was not measured in n=3 participants.

	Pregnancy cohort			Trophoblast cohort			PEACHES cohort		
	NGT	GDM	P	NGT	GDM	P	NGT	GDM	P
N	34	29	-	7	7	-	52	52	-
Maternal age [years]	31±5	34±4	0.3	-	-	-	33±4	33±4	0.6
Pre-pregnancy body mass index [kg/m²]	28.1±5.1	29.4±4.6	0.4	24.9±4.5	26.0±5.1	0.7	23.1±2.3	23.4±2.7	0.5
Mid-pregnancy body mass index [kg/m²]	27.0±6.0	30.6±5.6	0.4	-	-	-	24.9±2.6[a]	25.4±2.8[a]	0.3
Gestational age at delivery [week]	40.2±6.1	37.0±5.1	0.08	39.1±1.6	38.1±1.7	0.3	39.4±1.1	39.0±1.0	0.1
Fasting glucose [mmol/L]	4.4±0.3	4.8±0.5	**$3x10^{-5}$**	4.3±0.3	5.4±0.4	**0.008**	4.4±0.5	5.0±0.5	**$3x10^{-5}$**
1-h glucose [mmol/L] (75g OGTT)	7.33±1.3	10.5±1.3	**$6x10^{-14}$**	5.2±0.8	9.0±1.9	**0.002**	6.9±1.5	9.4±1.8	**$3x10^{-7}$**
2-h glucose [mmol/L] (75g OGTT)	6.12±1.1	8.42±1.0	**$6x10^{-13}$**	5.0±0.6	7.3±1.18	**0.009**	5.8±1.3	7.4±1.5	**$3x10^{-4}$**
1-h glucose [mmol/L] (50g GCT)							5.9±1.4 (N=28)	8.3±0.6 (N=2)	**0.02**
Smokers before pregnancy [N]	2	4	0.1	-	-	-	6	13	0.1
Spontaneous delivery [N]	12	4	0.1	4	3	0.6	32	33	0.8
Female neonates [N]	6	5	0.1	3	0	0.2	26	27	0.8

Placental lipid analysis

Multiple linear regression analysis of fatty acid concentrations of five lipid classes (cholesterol esters (CE), diglycerides (DG), PL, NEFA, and TG) from placental homogenates showed that GDM women have 1.5-fold increased levels of TGs in placental tissue compared to NGT women with similar BMI (P=0.04, Figure 4D). In placental tissue of GDM compared to NGT women, main SFA C16:0 and C18:0 were higher (P=0.02 and P=0.01, Figure 4E, supplemental Figure 2B, S.71). Furthermore, eicosatrienoate (C20:3-n6) and docosahexaenoate (C22:6-n3) showed statistical trends for reduced levels in GDM compared to NGT placental tissue (P≤0.1, Figure 4F). Similarly, reduced amounts of C20:3-n6 were found in the PL fraction in placental tissue of GDM women (P=0.04, supplemental Table 1, S.74). Furthermore, GDM-associated elevations of arachinate (C20:0, PL fraction), as well as of docosanoate (C22:0, DG fraction, P≤0.04, supplemental Table 1, S.74) were observed. Differences in CE and TG fractions were absent (P>0.05, supplemental Table 1, S.74). Again, quantification of placental lipids was conducted by Marketa Kovařova.

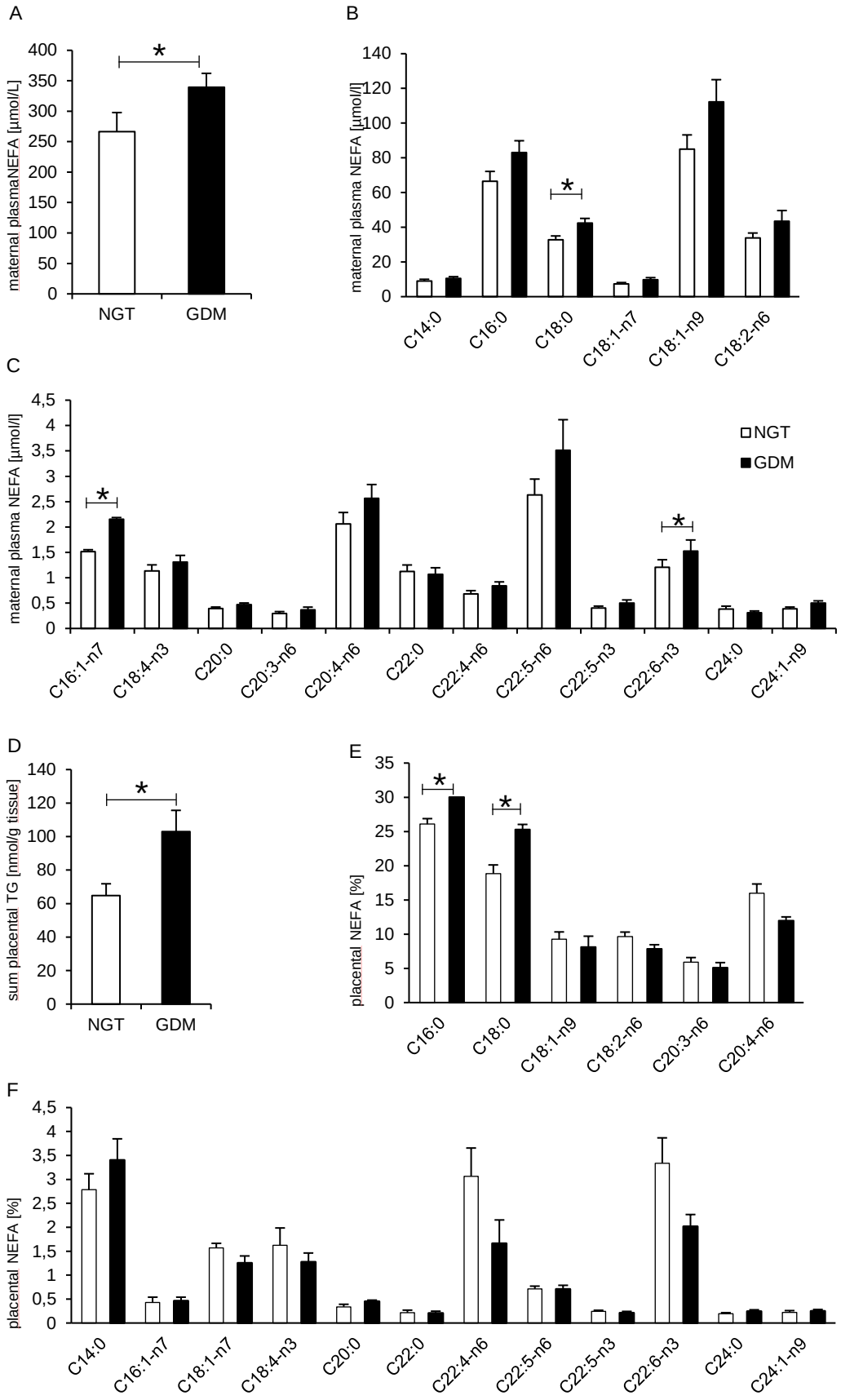

Figure 4: Lipid profiling (A) Sum of maternal plasma non-esterified fatty acids (NEFA) of normal glucose tolerant (NGT N=18, white columns) and gestational diabetes (GDM N=18, black columns) women with similar BMI. (B) Highly abundant (>5µmol/L) and (C) less abundant (≤5µmol/L) NEFA species of maternal plasma of NGT and GDM women. (D): Sum of placental triacylglycerol (TG) of NGT (N=8) and GDM (N=8) women with similar BMI. (E) Highly abundant (>5%) and (F) less abundant NEFA species (≤5%) of placental tissue of NGT and GDM women. Shown are means ± SEM. Multiple linear regression analysis (confounding covariates: BMI, gestational age, fetal sex) was conducted. Significant differences (P≤0.05) are marked with asterisks.

Gene expression of whole placental tissue

The impact of GDM on placental mRNA expression of genes involved in lipid metabolism (*FABP3, FABP4, FAT, LIPG, LPL, PLIN2, SLC27A1, and SLC27A4*) and inflammation (*IL1B, IL6, IL8, TNF-α, TLR2, TLR4*) was assessed. After multiple linear regression analysis (confounding covariates: BMI, gestational age, fetal sex), increased mRNA expression of *FABP3* (fold change (FC)=1.4; P=0.01, Figure 5A), *IL1B* (FC=1.6; P=0.03, Figure 5B), *IL6* (FC=2.0, P=0.02, Figure 5C), and *TLR2* (FC=1.1; P=0.03, Figure 5E) was detected in placental tissue of GDM women. Furthermore, a statistical trend for elevated *IL8* (P=0.08, Figure 5D) mRNA expression in tissue of GDM women was observed. No further differences for other genes investigated were identified (P>0.1, supplemental Figure 3, S.72) As the chemokine *IL8* plays a major role in mediation of immune cell infiltration [107] and its GDM-associated expression has been shown earlier [108], we included it in further analyses.

Additionally, we correlated maternal plasma total NEFA values with placental mRNA expression. Significant positive correlations of maternal total plasma NEFA concentrations and placental *IL1B*, *IL6*, and *TLR2* mRNA expression were found (P≤0.05, Figure 5F, G, I). For placental *IL8* mRNA, the association with maternal plasma NEFA concentrations was of borderline significance (P=0.08, Figure 5H). Multiple linear regression analysis demonstrated significantly elevated protein levels of IL1B and IL6 (P=0.03, Figure 5J, K) and statistical trends for elevated IL8 (P=0.08, Figure 5L) and TLR2 levels (P=0.07, Figure 5M, supplemental Figure 4, S. 72) in placental tissue of GDM women.

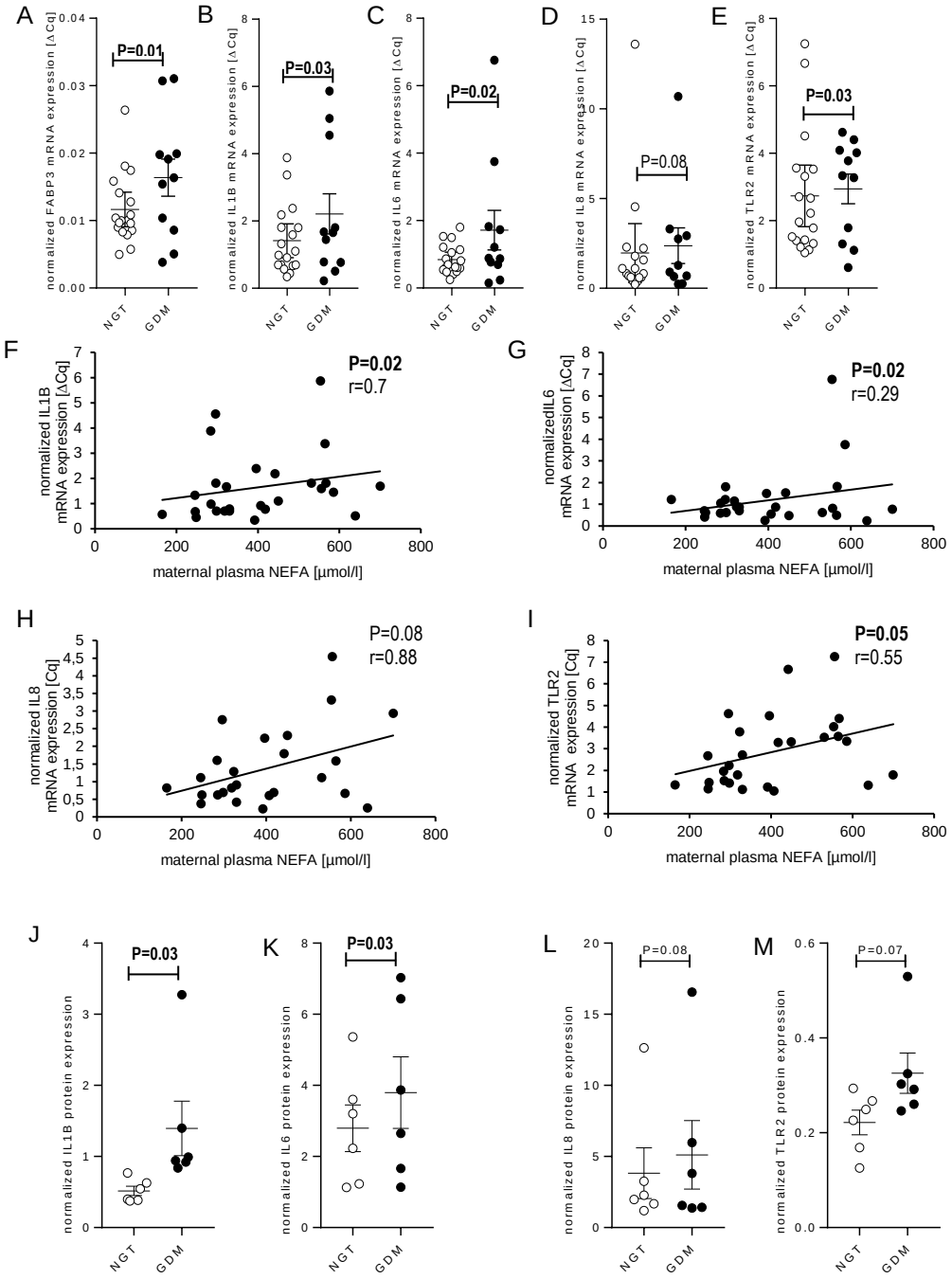

Figure 5: Gene expression in whole placental tissue. (A-E) mRNA expression of *FABP3, IL1B, IL6, IL8, PLIN2,* and *TLR2* in placental tissue of normal glucose tolerant (NGT N=19) and gestational diabetes (GDM N=11) women. (F-I) Correlation of maternal plasma NEFA with placental mRNA expression of *IL1B, IL6, IL8*, and *TLR2*. Regression coefficient (r) and P-value are shown. (J-M) Protein expression of *IL1B, IL6, IL8* and *TLR2* in placental tissue of NGT (N=6) and GDM (N=6) women with similar BMI. Individual data ± SEM are shown. Significant differences (P≤0.05) detected by multiple linear regression analysis (confounding covariates: BMI, gestational age, fetal sex) are indicated in bold.

Gene expression of NEFA-stimulated primary trophoblasts derived from NGT and GDM women

Based on the findings in whole placenta, mRNA expression of *FABP3, IL1B, IL6, TLR2,* and *PLIN2* was quantified in primary human trophoblasts derived from GDM and NGT women with similar BMI. After isolation from placenta, the trophoblasts were cultured in medium for 48h. Elevated mRNA expression for *IL6* (N=12), *IL8* (N=12) and *TLR2* (N=10) (P≤0.04, all, Figure 6A-C) and a statistical trend for elevated expression of *PLIN2* (N=12, P=0.08, Figure 6D) were found in trophoblasts derived from GDM women (reduced group sizes due to

individual C_q values>35). *FABP3* (N=8) mRNA did not differ between both groups (P=0.3, Figure 6E). *IL1B* mRNA expression signals were at the detection limit (C_q values≥35) and, hence, excluded from further analysis.

As we found elevated concentrations of palmitate (C16:0) and stearate (C18:0) and altered mRNA expression in whole placental tissue of GDM women, we investigated possible lipoinflammatory effects of defined NEFA on trophoblast mRNA expression. Therefore, primary human trophoblasts derived from GDM women and NGT controls were stimulated with the SFA C16:0 and the monounsaturated NEFA oleate (C18:1 n-9), respectively, as well as a control condition. SFA-mediated activation of TLR-signaling was shown earlier to contribute to an elevated mRNA expression of cytokines or chemokines (lipoinflammation) [59, 109]. In contrast to that, stimulation with the unsaturated fatty acid such as oleate was not associated with lipoinflammation. Therefore, we choose oleate as a non-lipotoxic control NEFA [59, 110]. As in placental tissue and non-manipulated trophoblasts, mRNA expression of *FABP3, IL6, IL8, PLIN2,* and *TLR2* was analyzed in NEFA-stimulated trophoblasts. After stimulation with palmitate, we found increased mRNA expression of *IL6, IL8, PLIN2*, and *TLR2* (P≤0.05, Figure 6F). *FABP3* mRNA expression was unchanged (P=0.9, Figure 6F). Differences of mRNA expression after stimulation with palmitate were also observed on protein level: a statistical trend for elevated IL6 concentration in cell culture supernatant was observed (P=0.09, Figure 6H). In contrast, stimulation with oleate resulted in no different mRNA and protein expression (P≤0.05, Figure 6G, protein data not shown). Quantification of IL1B levels in the cell culture supernatant was not possible, as concentrations were below the detection limit.

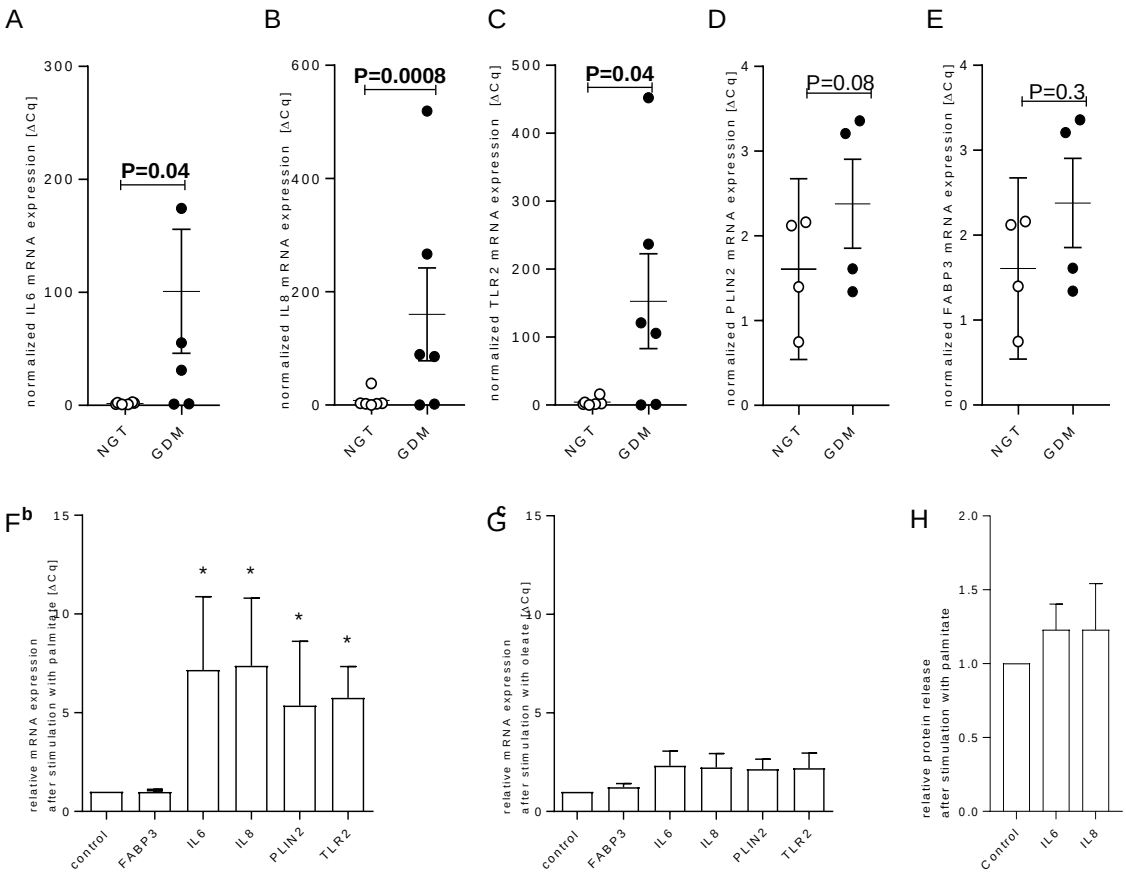

Figure 6: Gene expression of inflammatory genes and of genes involved in fatty acid binding and storage in trophoblasts: (A-E) *IL6, IL8, TLR2, PLIN2*, and *FABP3* mRNA expression of primary trophoblasts (N≥8) after 48-h incubation with medium only. Expression is given as fold change with respect to the BSA control. P-values of a one-tailed Student's t-test are indicated. mRNA expression of primary trophoblasts (N=14) after 24-h stimulation with (F) 100µM palmitate or (G) 100µM oleate. (H) Amount of IL6 and IL8 in supernatants of primary trophoblasts after 24-h stimulation with 100µM palmitate relative to corresponding BSA controls. Significant differences (P≤0.05) are indicated by asterisks show the results of analysis of variance (ANOVA) followed by Dunnett's test. Mean ± SEM are shown.

Quantification of CD68-positive cells in placental tissue of NGT and GDM women

To understand whether NEFA-mediated differences in trophoblastic chemokine and cytokine release may have a biological consequence within the placenta we compared the number of placental villus macrophages in NGT vs. GDM women. In placental tissue collected from NGT women a weak (Figure 7A) and in placental tissue of GDM women a strong (Figure 7B) CD68 staining was observed. Linear regression analysis revealed a statistical trend for elevated numbers of CD68-positive villi within the placenta of GDM compared to NGT women (P=0.06, Figure 7C).

Cord blood lipid profiles and cytokine patterns

Inflammatory patterns and NEFA profiles of neonates from GDM and NGT women with similar BMI were determined to investigate if placental lipoinflammation has any consequences for fetal metabolism. We quantified inflammatory parameters in neonatal cord blood. Significantly reduced concentrations of eotaxin, fibroblast growth factor (FGF)-2, IL4, IL9, IL17, interferon gamma-induced protein (IP-10), macrophage inflammatory protein (MIP-1β), and tumor necrosis factor (TNF-α) were found in cord blood samples from GDM women

(P≤0.05, Figure 7D) after multiple linear regression analysis. In groups with smaller sample sizes (concentrations outside the standard curve excluded), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL6, and IL15 levels were reduced (Figure 7D). No differences for other inflammatory parameters were present (data not shown, P>0.1). Furthermore, we could detect statistical trends for reduced levels of SFA C18:0, C20:0 and C24:0 in the NEFA fraction of cord blood from GDM offspring in comparison to controls (P≤0.1, Figure 7E, F and supplemental Figure 2, S.71). In the DG fraction significant GDM-associated elevation of linoleate (C18:2-n6) and C20:3-n6 and reduction of myristate (C14:0) were seen (P≤0.05, data not shown). In the CE, PL, as well as TG fractions no differences in fatty acid profiles were observed (P>0.05, data not shown).

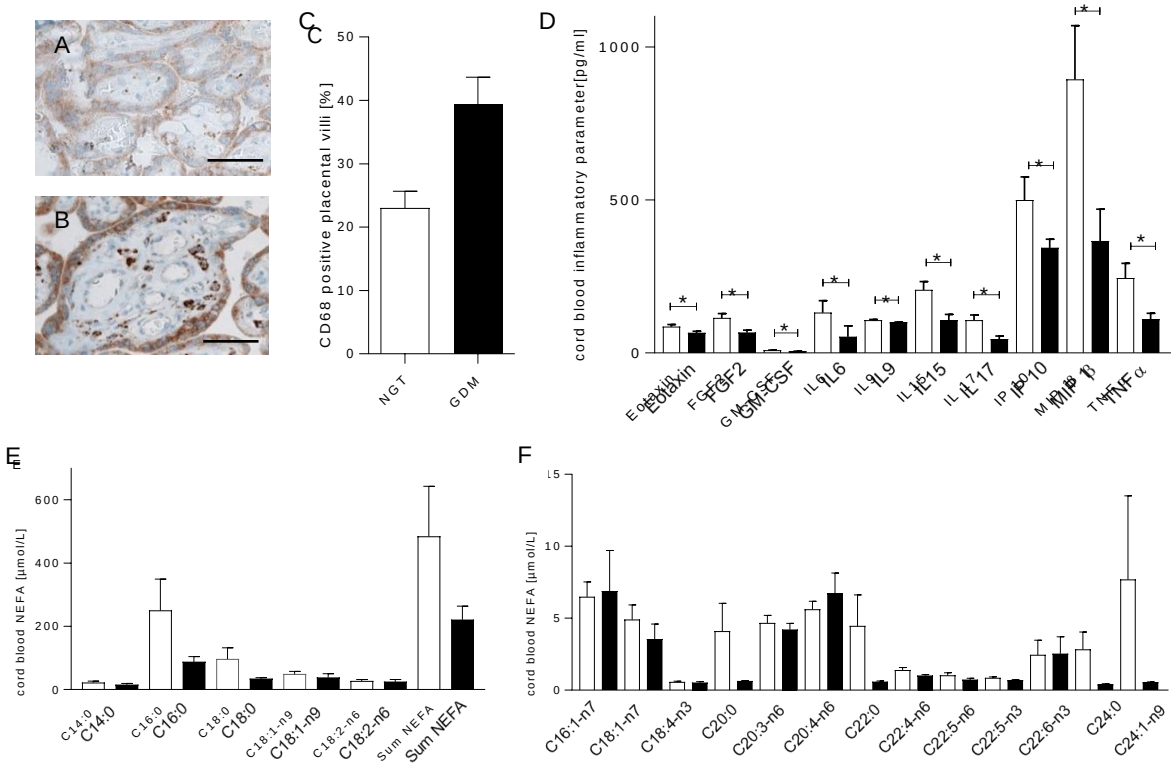

Figure 7: Biological relevance of placental lipoinflammation. Staining of placental tissue derived of (A) a normal glucose tolerant (NGT) and (B) a gestational diabetic (GDM) woman with antibodies against CD68 (brown). Representative images; bar: 50µm. (C) Percentage of CD68-positive villi in tissue of GDM women (N=3) and NGT controls (N=3) with similar BMI. (D) Concentrations of significantly different inflammatory parameters in cord blood samples of GDM women (N=52) and NGT controls (N=52) with similar BMI. Cord blood concentrations of (E) highly abundant (>10µmol/L) and (F) low abundant (<10µmol/L) non-esterified fatty acids (NEFA) pattern in NGT (N=11) and GDM women (N=7). Multiple linear regression analyses (confounding covariates: BMI, gestational age, fetal sex) were conducted. Statistically significant differences (P≤0.05) are marked by asterisks.

Project II: BMI-independent effects of maternal GDM on placental apelinergic system

In response to GDM-associated differences of placental lipid profiles, expression of placental hormones could also be changed and contribute to fetal programming.

GDM effects on placental and trophoblast *APL* and *APJ* (APL receptor) gene expression have not been studied yet. Furthermore, no relevant information of potential GDM-associated differences of fetal APL plasma concentrations is currently available. Therefore, the scientific goal of Project II was to investigate BMI-independent GDM-associated expression differences of the placental apelinergic system and its regulation by GDM-associated metabolic stimuli. Finally, to see if GDM-associated placental *APL* expression differences may directly influence fetal metabolism, cord blood APL levels of NGT and GDM were quantified and compared.

Characterization of pregnant women and placental lipid analysis are shown in Project I.

Placental APJ and APL gene expression

Applying multiple linear regression analysis, significantly elevated mRNA expression of *APL* in placental tissue of GDM women was found (P=0.006, Figure 8A). APL protein expression was not different (P=0.8, data not shown). In comparison to NGT women, a statistical trend for elevated *APJ* mRNA expression in placental tissue of GDM women was found (P=0.09, Figure 8B). Quantification of APJ protein expression revealed a significantly elevated expression in GDM women (P=0.03, Figure 8C and D). As placental tissue consists of several cell types (i.e., mesenchymal cells, fibroblasts and macrophages, and (syncytio-) trophoblasts), potential cellular sources of APL and APJ expression were investigated. Immunohistochemical staining of placental slides indicated APL- and APJ-positive staining of the outmost layer of placental villi (syncytiotrophoblasts, Figure 8E and F) in comparison to isotype control (Figure 6G).

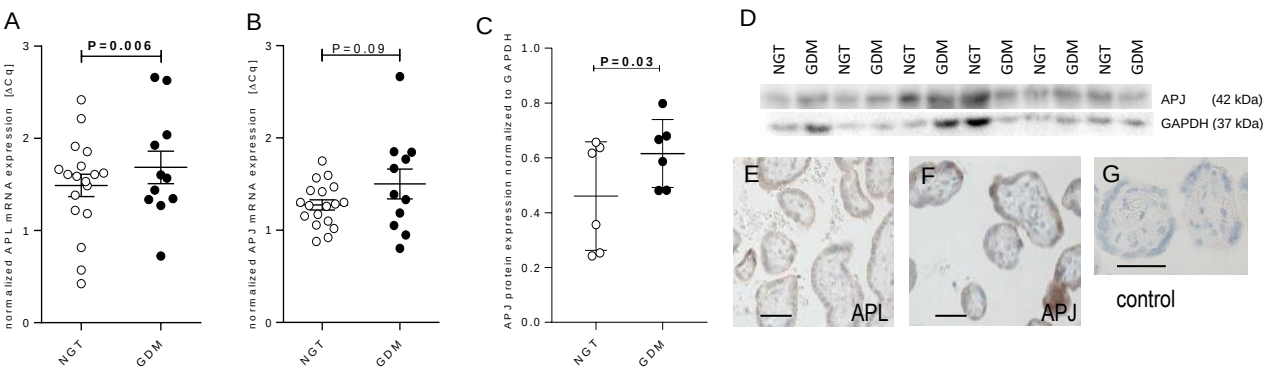

Figure 8: APL (apelin) and APJ (apelin receptor) expression in placental tissue. Relative placental (A) *APL* and (B) *APJ* mRNA expression of NGT (N=19) and GDM (N=11) women. (C) Normalized APJ protein expression of GDM (N=6) and BMI-matched NGT (N=6) women. Significant (P≤0.05) changes are indicated in bold and show the results of a multiple linear regression model (confounding covariates: BMI, gestational age, and fetal sex) Mean ± SEM are shown. (D) Western Blot shows the protein expression of GAPDH and APJ in GDM and NGT women with similar BMI. Immunohistochemical staining of placental tissue slides against (E) APL, (F) APJ, and (G) isotype control. Positive staining is indicated in brown. Representative images; bar: 50µm.

Association of placental APJ expression with placental NEFA content

As shown in Project I, GDM-associated elevations of both SFA C16:0 and C18:0 were found in placental tissue. To investigate if placental NEFA are associated with placental *APJ* mRNA expression, linear regression analysis was conducted. A significant positive correlation was found for placental palmitate content and placental *APJ* mRNA expression (Figure 9A, P=0.01). Furthermore, a statistical trend for positive correlation was found between placental stearate content and placental *APJ* mRNA expression (P=0.08, Figure 9B), only. No correlation of placental NEFA with placental *APL* mRNA expression was seen (P≥0.9, data not shown).

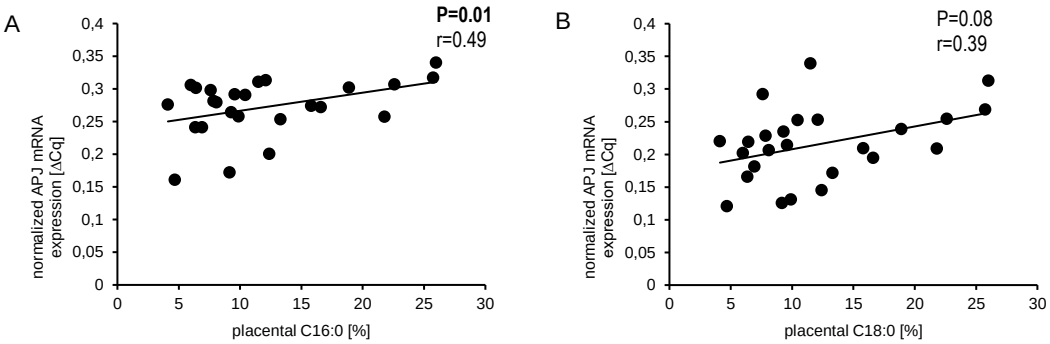

Figure 9: Correlation of placental NEFA and placental APJ mRNA expression. Correlation of placental (A) palmitate (C16:0) and (B) stearate (C18:0) contents with placental *APJ* mRNA expression (N=30). P-value and correlation coefficient of linear regression (r) are shown. Significant (P≤0.05) correlations are indicated in bold.

Gene expression of NEFA stimulated primary trophoblasts

Trophoblasts were isolated from NGT and GDM women with similar BMI. *APL* as well as *APJ* mRNA expression was compared between both groups. We could show that primary trophoblasts from GDM women express elevated *APJ* mRNA levels (P=0.03, Figure 10A). *APL* mRNA expression could not be quantified (C_q-value>35). Next, primary trophoblasts isolated from NGT and GDM women were stimulated with palmitate, oleate, insulin and corresponding controls. Stimulation with palmitate led to a significantly elevated expression of *APJ* (P=0.002, Figure 10B). Furthermore, primary cells derived from GDM women showed a stronger response of *APJ* expression after stimulation with palmitate (P=0.03, Figure 10C). Stimulation with oleate led to a statistical trend for elevated *APJ* mRNA expression (P=0.09, Figure 10B). Stimulation with insulin was associated with a statistical trend for reduced *APJ* gene expression (P=0.08, Figure 10B). Additionally, we collected cell culture supernatant and quantified APL protein release of cultured primary trophoblasts. Trophoblasts isolated from GDM women released more APL compared to NGT women with similar BMI (P=0.03, Figure 10D). Stimulation of trophoblasts with insulin, palmitate and oleate was not associated with differences in APL release in cell culture supernatant (P>0.3, data not shown).

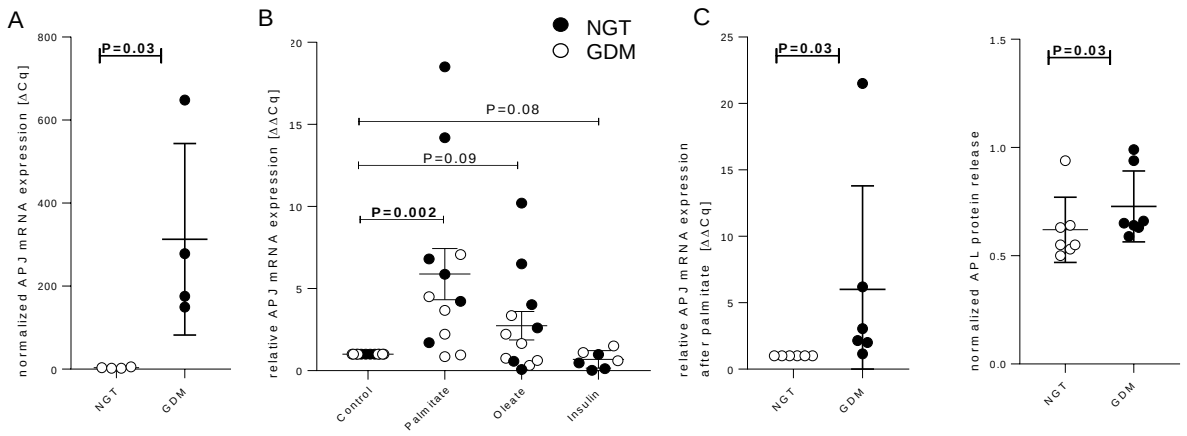

Figure 10: Gene expression of primary trophoblasts stimulated with palmitate, ‹ *Apelin receptor* (*APJ*) mRNA expression of primary trophoblasts (N=8) after 24-h incubation with medium only. (B) *APJ* expression after stimulation with 100µM palmitate, or oleate, or 10nM insulin (N≤7). Expression is given as fold change with respect to the BSA control. (C) Comparison of *APJ* mRNA expression after stimulation with palmitate between trophoblasts collected form NGT and GDM women. (D) Concentrations of apelin (APL) in cell culture supernatant of primary trophoblasts after 24-h culture in medium. Significant (P≤0.05) differences are indicated in bold and show the results of a one-tailed Student's t-test. Mean ± SEM are shown.

Fetal APL cord blood concentrations

To investigate if GDM-associated differences in the placental apelinergic system have any impact on fetal metabolism, APL cord blood levels were quantified. After applying multiple linear regression analysis, no differences in APL cord blood levels collected from offspring of NGT and GDM women were found (N=222, PEACHES, P=0.2). As plasma APL concentrations were shown to be different in women and men, we stratified the data for fetal sex [111]. In cord blood samples of female offspring from GDM pregnancies, we observed a statistical trend for elevated APL cord blood concentrations (N=102, P=0.10, Figure 11 and Table 2). In cord blood of male offspring, no differences were seen (N=106, P=0.4, data not shown).

Table 2: Raw APL cord blood concentrations in male and female offspring of NGT and GDM women. Data are given as absolute numbers and means ± SD.

	Male offspring		Female offspring	
	NGT	GDM	NGT	GDM
N	53	56	54	52
Mean [ng/ml]	1.54±1.05	1.24±0.59	1.22±0.50	1.38±0.58

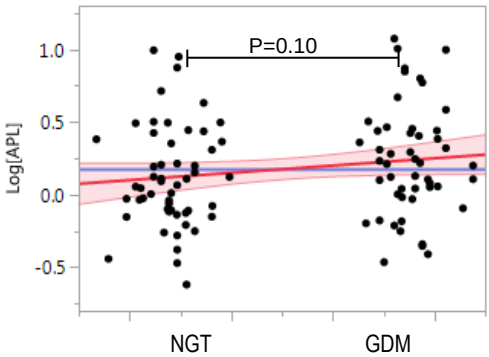

Figure 11: Adjusted cord blood apelin (APL) concentrations in female offspring of NGT and GDM women. Adjusted concentrations of APL in fetal cord sera of NGT (N=54) and GDM (N=52) women with similar BMI. After multiple linear regression analysis (confounding covariates: BMI, gestational age), no statistically significant differences were seen.

Project lll: GDM-associated alterations of maternal whole blood miRNA patterns

It is hypothesized that epigenetic changes occurring in response to GDM can contribute to progression of GDM and adverse fetal programming Furthermore, miRNA expression differences in easily accessible compartments can serve as future indicators for diabetes.

In the first part of Project lll, GDM-associated WBC non-coding RNA patterns were investigated. Additionally, we determined which metabolic stimuli can cause similar transcriptional differences in lymphocytes, *in vitro*.

In the second part of Project lll, maternal GDM-associated cell-free miRNA patterns were characterized. As plasma miRNAs were shown to be taken up by cells and contribute to regulation of gene expression and metabolism there [112], GDM-associated expression differences can have relevance for placental [113] and fetal [114] metabolism.

Patient characteristics

The workflow of the study including the criteria for participant selection of the screening, the validation, the lymphocyte donor populations as well as the cord blood population are shown in Figure 12. The clinical characteristics of the pregnant NGT (N=8) and GDM (N=8) women of the WBC screening group are shown in Table 3. Both groups did not significantly differ in maternal age, BMI during pregnancy, family history of parental diabetes as well as time-point of blood sampling and weight gain during pregnancy ($P \geq 0.2$, Table 3). Women with GDM had significantly increased 1-h glucose and fasting insulin values and decreased insulin sensitivity index (ISI, $P \leq 0.05$). Statistical trends for differences were observed in fasted and 2-h glucose levels of the OGTT ($P \leq 0.1$). Similar associations were found in pregnant women of the validation and lymphocyte donor groups (Table 3). Anthropometric characteristics of the children (birth size, birth weight and sex) and of the non-pregnant controls (age, BMI) are also given in Table 3. For the plasma miRNA study, samples of NGT (N=9) and GDM (N=9) women were collected. Plasma of all participants of the WBC screening cohort was included. Characteristics of the plasma miRNA group are shown in Table 4 and are similar to those of the screening group. Anthropometric and metabolic characterization of the participants was conducted by the physicians and clinical scientists of the Clinical Study Center of the Department of Internal Medicine, Division of Endocrinology, Diabetology, Angiology, Nephrology and Clinical Chemistry, University Hospital Tübingen.

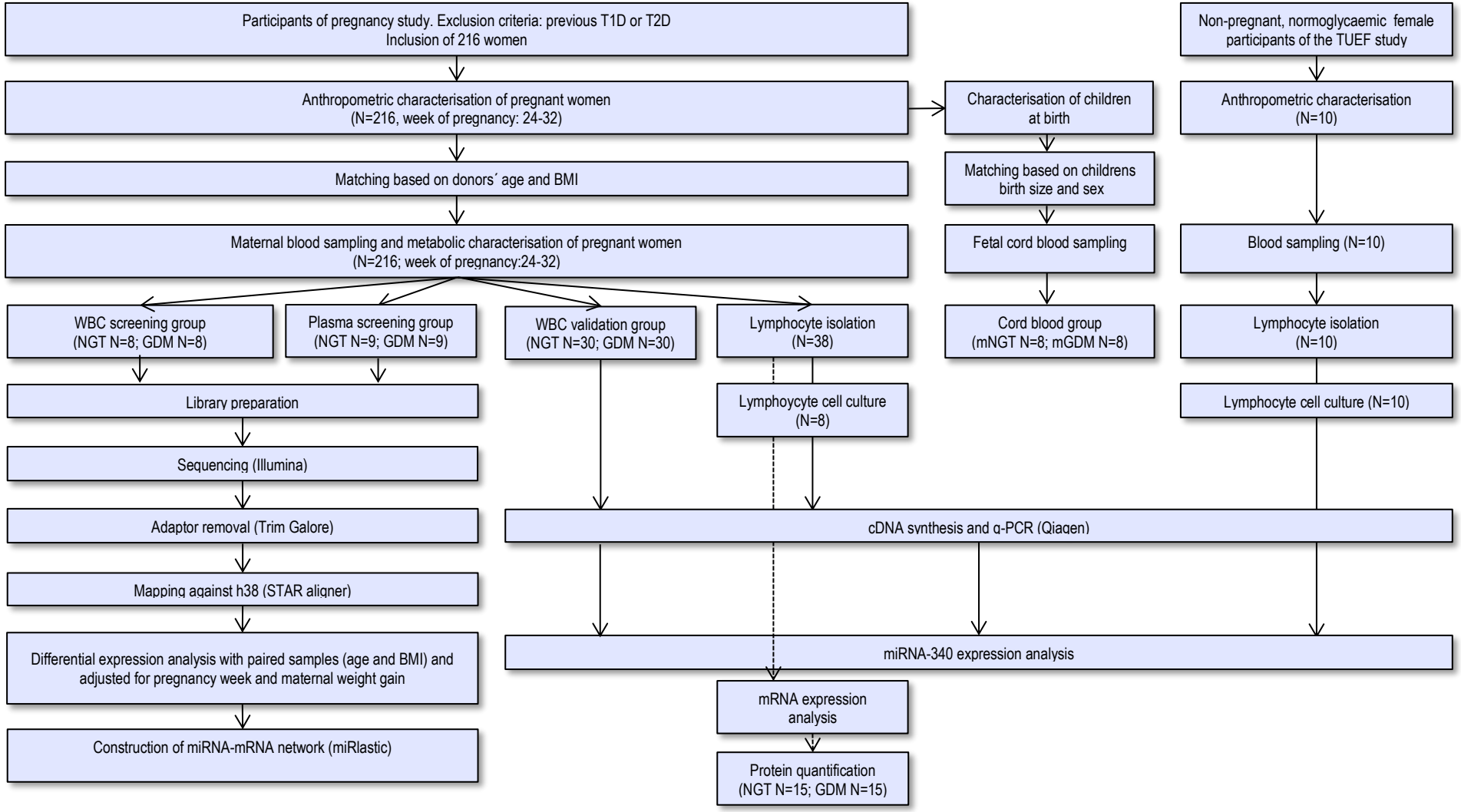

Figure 12: Criteria for participant selection and work flow. Whole blood was collected from normal glucose tolerant (NGT N≤9) and gestational diabetes (GDM N≤9) pregnant women for genome-wide transcriptome analysis of miRNAs isolated from whole blood cells (WBC) or plasma (plasma screening group). Samples were matched based on age and body mass index (BMI) and were derived from the pregnancy cohort. After whole blood RNA isolation, sequencing (Illumina), library preparation and biostatistical analysis (miRlastic) was conducted. Next, for qPCR validation of miRNA expression of WBC, NGT (N=30) and GDM (N=30) women were selected (WBC validation group). Additionally, quantification of *miRNA-340* and target mRNAs was conducted in lymphocytes collected from pregnant NGT and GDM women (N=38, lymphocyte donors). From this group either lymphocyte cell culture (N=8) or protein quantification was conducted (N=30). Additionally, cord blood samples were collected from children from NGT pregnancies (mNGT N=8) and from GDM pregnancies (mGDM N=8). Finally, cell culture and *miRNA-340* quantification was conducted in lymphocytes of non-pregnant controls (N=10). Figure adopted from [1]

Table 3: Anthropometric and metabolic characteristics of different participant groups. All data are given as absolute numbers or means ± SD. Significant differences calculated with a two-tailed Student's t-test or Chi-square-test (family history of diabetes) are marked by using bold fonts. Missing values are indicated with a minus. Blank table cells refer to not applicable data.

	Screening group			Validation group			Cord blood group			Lymphocyte donors			Non-pregnant controls
	NGT	GDM	P	NGT	GDM	P	mNGT	mGDM	P	NGT	GDM	P	NGT
N	8	8	-	30	30	-	8	8	-	20	18	-	10
Age [years]	33±5	32±3	0.7	32±4	31±4	0.4				32±4	31±4	0.3	35±8
Body mass index [kg/m^2]	28.1±5.4	29.1±6.1	0.7	29.5±5.6	29.8.2±4.07	0.7				27.6±4.0	28.0±4.2	0.2	22.5±3.0
Pregnancy week [weeks]	23.0±9.5	25.9±1.7	0.4	27.6±2.37	27.0±2.3	0.4				26.5±2.0	24.8±7.2	0.3	-
Fasting glucose [mmol/L]	4.44±0.27	4.89±0.62	0.09	4.58±0.3	4.87±0.47	**0.005**				4.43±0.21	4.79±0.51	**0.01**	-
1-h glucose [mmol/L]	7.76±1.21	10.90±0.82	**0.00013**	7.97±1.64	10.41±1.52	**0.0001**				8.17±1.08	10.79±1.01	**0.0003**	-
2-h glucose [mmol/L]	6.32±1.31	7.52±1.44	0.1	6.36±1.27	7.94±1.71	**0.002**				6.49±1.06	7.83±1.5	**0.01**	-
Fasting insulin [pmol/L]	59.88±28.30	126.38±53.70	**0.01**	68.71±28.15	116.76±44.99	**0.006**				68.71±28.15	116.76±45.0	**0.006**	-
Insulin sensitivity index [10^{19} L^2/mol^2]	13.6±6.8	5.0±2.0	**0.03**	9.63±4.83	5.46±2.25	**0.00005**				5.54±2.02	7.92±6.43	0.1	-
Maternal weight gain [kg]	6.53±3.38	11.27±9.64	0.2	6.91±3.53	8.52±6.39	0.9				7.37±5.64	7.39±9.64	0.4	
Family history of parental diabetes	Paternal = 0 Maternal = 0	Paternal = 1 Maternal = 0	0.5	Paternal = 0 Maternal= 0	Paternal = 4 Maternal = 1	0.3				Paternal = 0 Maternal= 0	Paternal = 2 Maternal =1	0.8	
Birth weight [kg]							3.38±0.59	3.36±0.51	0.9				
Birth size [cm]							51.2±1.6	51.0±2. 6	0.8				
Fetal sex [female]							4	4	-				

Table 4: Anthropometric characteristics of plasma miRNA screening group. All data are given as absolute numbers or means ± SD. Group differences were calculated by two-tailed Student's t-test.

	NGT	GDM	P
N	9	9	-
Age [year]	34.44±3.80	34.44±4.31	1
BMI [kg/m2]	28.70±5.70	27.82±4.26	0.2
Pregnancy week of blood collection [week]	26.56±2.30	26.0 ±1.60	0.6
Mid-pregnancy weight gain [kg]	6.57±2.79	7.13±4.05	0.8

miRNA expression patterns in WBC related to maternal GDM

To identify differential coding and non-coding RNA expression in WBC between GDM and NGT women with similar BMI, RNA sequencing was conducted in the WBC screening group. Special focus was on the comparison of the expression of miRNAs, as approximately 80% of the identified unique mappings among the non-coding RNA species were miRNAs (data not shown). The participants were selected based on maternal age and BMI. Next, data was adjusted for maternal weight gain and gestational age. Twenty-nine miRNAs were found to be elevated in WBC of GDM women ($P<0.05$; false discovery rate [FDR]<0.1, Table 5). No GDM-related reduction in miRNA expression was detected. PIWI-interacting RNAs (piRNA) and transfer RNA (tRNA) expression was not different between both groups. Non-coding RNA sequencing was conducted by Peter Huypens, Institute of Experimental Genetics, Helmholtz Center Munich. Bioinformatic analysis was conducted by Steffen Sass, Institute of Computational Biology, Helmholtz Center Munich.

Next, miRNA sequencing results were validated by quantitative polymerase chain reaction (qPCR) validation experiments in a lager WBC validation group (NGT N=30, GDM N=30). We selected five miRNAs (*miRNA-19a, -142, -143, -340,* and *let-7g*) with the highest FC and with interaction sites within mRNAs negatively associated with GDM in the screening population. Additionally, *miRNA-19b* was included in qPCR validation, due to its proposed relevance in the network analysis (Figure 14). The six miRNAs selected for qPCR validation are marked in Table 5 by bold fonts. After q-PCR validation, *miRNA-19a*, and *-19b* were not significant differently expressed in WBC between GDM and NGT ($P\geq0.3$, both, Figure 13A, B). For *miRNA-142, miRNA-143,* and *let-7g,* statistical trends for elevated expression in GDM women were observed ($0.06\leq P\leq0.1$, Figure 13 C-E). Out of six miRNAs selected for validation, *miRNA-340* was the only one found to be significantly elevated in GDM ($P=0.03$, Figure 13F).

Table 5: MiRNAs positively associated with maternal GDM in whole blood cells of the screening group. Table 5 shows miRNAs significantly (P<0.05; false discovery rate (FDR)<0.1) associated with GDM in the screening group. The samples were paired based on maternal age and BMI and adjusted for maternal weight gain and pregnancy week. GDM/NGT was defined as endpoint variable. miRNAs are sorted by fold changes (FCs), shown in the 2nd column. Logarithmically transformed counts per million (logCPM), indicating the relative abundance of the transcript are shown in the 3rd column of the table. P-values and FDR are indicated in the 4th and 5th column, respectively. MiRNAs shown in bold fonts indicate those which were selected for qPCR validation experiments.

Name	FC	logCPM	P	FDR
miRNA-199a-3p	2.213	3.917	7.50E-03	0.083
miRNA-199b-3p	2.213	3.917	7.50E-03	0.083
miRNA-15a-5p	2.169	9.190	4.88E-03	0.072
miRNA-19a-3p	2.154	5.782	1.48E-03	0.035
miRNA-96-5p	2.036	7.029	1.65E-04	0.020
let-7g-5p	1.965	12.247	4.32E-04	0.024
miRNA-143-3p	1.946	7.242	8.72E-04	0.028
miRNA-340-5p	1.907	6.828	7.06E-04	0.028
miRNA-142-5p	1.882	11.583	3.36E-04	0.024
miRNA-1307-5p	1.870	5.413	1.04E-03	0.028
let-7f-5p	1.836	11.930	5.32E-03	0.072
miRNA-107	1.825	9.919	6.44E-04	0.028
let-7c-5p	1.749	4.566	1.81E-04	0.020
miRNA-18a-5p	1.735	4.410	8.56E-03	0.088
miRNA-660-5p	1.730	6.842	2.15E-03	0.041
miRNA-17-3p	1.728	5.856	2.15E-04	0.020
miRNA-19b-3p	1.629	9.256	5.28E-03	0.072
miRNA-106b-5p	1.617	8.228	5.65E-03	0.073
let-7a-5p	1.611	12.127	1.10E-03	0.028
miRNA-451a	1.606	17.754	3.96E-03	0.067
let-7e-5p	1.589	3.544	8.35E-03	0.088
let-7i-5p	1.589	11.408	6.85E-03	0.082
miRNA-148a-3p	1.584	11.695	4.21E-03	0.067
miRNA-22-3p	1.577	13.539	9.01E-04	0.028
miRNA-17-5p	1.571	9.101	9.47E-03	0.094
miRNA-145-5p	1.569	4.349	6.23E-03	0.078
miRNA-93-5p	1.546	10.233	3.88E-03	0.067
miRNA-103a-3p	1.495	12.179	1.75E-03	0.035
miRNA-103b	1.495	12.179	1.75E-03	0.035

mRNA expression patterns in WBC related to maternal GDM

Similarly, mRNA sequencing was conducted within the WBC screening population (Peter Huypens) and the dataset was examined (Steffen Sass). One hundred sixty-three mRNAs were significantly (P<0.05; FDR<0.1) reduced in GDM in the WBC screening group (P<0.05; FDR<0.1, supplemental Table 2, S.74). No GDM-related elevation of mRNA was detected. For mRNA qPCR validation, predicted targets of *miRNA-340*, shown to be negatively related with GDM (P<0.05; FDR<0.2), were selected. Different group sizes of the validation groups result from limited sample amount (N=60 for *CRY2, GRB10, PAIP1*; N=48 for *ID2, PITPNB, SCARB2,* and *SPRY3*). GDM-related significant reductions in expression were observed for *GBR10* (P=0.05, Figure 13H) and *PAIP1* (P=0.007, Figure 13M). For *CRY2, ID2, PITPNB, SCARB2,* and *SPRY3* no expression differences related to GDM were found (Figure 13 G, I, J-L).

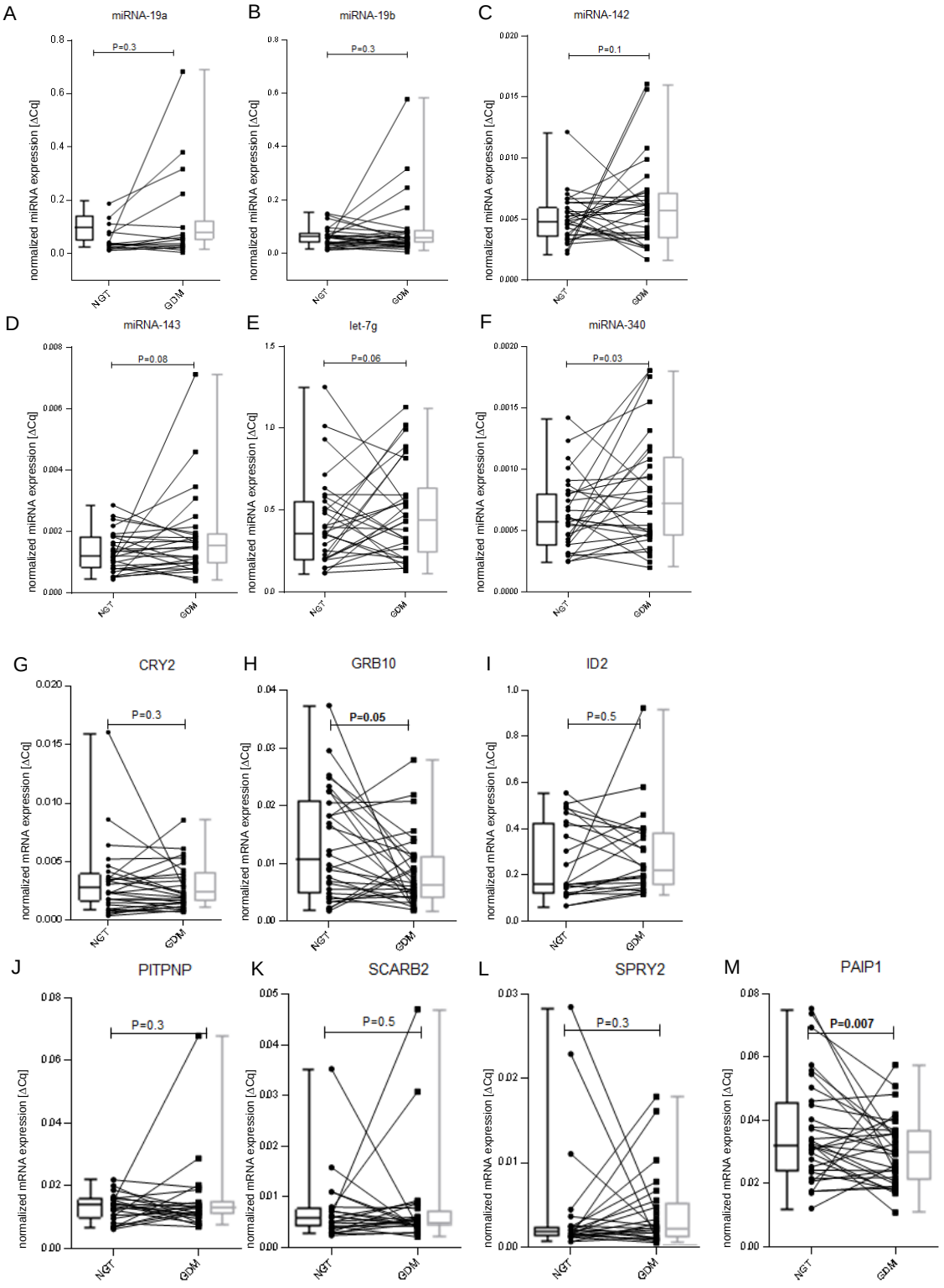

Figure 13: Validation of miRNA and mRNA sequencing results within the validation group by qPCR. Sequencing results were validated within the validation group of normal glucose tolerant (NGT N=30) and gestational diabetes (GDM N=30) pregnant women by qPCR. (A-F) Six miRNAs were selected for qPCR validation experiments within the validation. (G-M) Seven mRNAs, which are all predicted targets of *miRNA-340* were selected for qPCR validation experiments. Samples were paired based on maternal age and body mass index. Data was adjusted for pregnancy week and weight gain till the end of second trimester. Shown are the means ± SD. Differences considered as statistically significant (P≤0.05) are marked by bold fonts. Figure adopted from [1]

Results of the WBC screening and WBC validation experiments are displayed in the network analysis generated with miRlastic R by Steffen Sass. Figure 14 shows each GDM-related miRNA in the center of the cluster, surrounded by their mRNA targets negatively related with GDM within our dataset (P<0.05, FDR<0.1).

miRNA-340 and *GRB10* and *PAIP1* mRNA and protein expression in lymphocytes of NGT and GDM women

Primary lymphocytes were collected from participants (NGT N=15, GDM N=15) in order to see if GDM-related miRNA and mRNA expression is similar to the GDM-related expression in WBC. Expression analysis of *miRNA-340* and both predicted targets *GRB10* and *PAIP1* were conducted. In lymphocytes of GDM women, significantly elevated *miRNA-340* expression was seen (P=0.02, Figure 15A). Furthermore, a statistical trend for reduced *GRB10* mRNA expression was observed in lymphocytes of GDM women (P=0.1, Figure 15B). Differences for *PAIP1* mRNA expression between both groups were absent (P=0.3, Figure 15C). As miRNAs can influence target gene expression via mRNA degradation and inhibition of protein translation, expression of GRB10 and PAIP1 was quantified on protein level, additionally [115]. Relative protein quantification by Western blots indicate, that GRB10 protein expression was not different between both groups (P=0.7, supplemental Figure 5A, B, S.73). For PAIP1 a statistical trend for reduced protein expression was observed after normalization with the reference protein GAPDH (P=0.06, supplemental Figure 5A, C, S.73). To investigate if *miRNA-340* expression has any association with PAIP1 mRNA or protein expression, a regression analyses conducted. We found no significant correlation between *miRNA-340* and *PAIP1* mRNA expression (N=30; P=0.3, Figure 15D). However, a significant negative correlation of *miRNA-340* with PAIP1 protein expression (N=30; P=0.006, Figure 15E) was observed. Batch correction was included in the analysis, as RNA isolation of lymphocytes was conducted in two independent experiments.

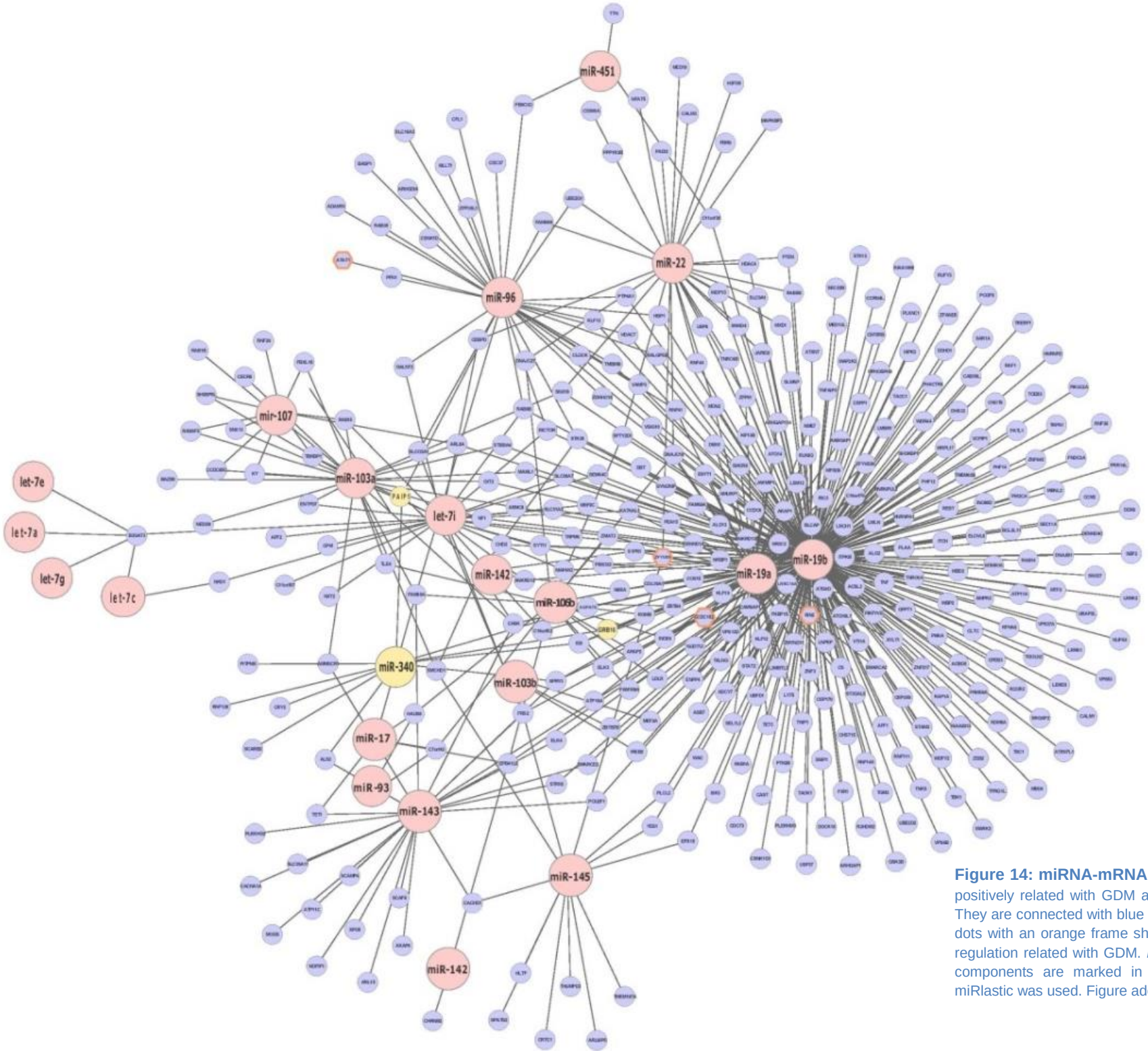

Figure 14: miRNA-mRNA regulatory network inference. MiRNAs positively related with GDM are indicated as red dots (P<0.05; FDR<0.1). They are connected with blue dots, indicating predicted target mRNAs. Blue dots with an orange frame showed a significant (P<0.05; FDR<0.2) down-regulation related with GDM. *MiRNA-340, GRB10,* and *PAIP1* as validated components are marked in yellow. For data analysis the R package miRlastic was used. Figure adopted from [1]

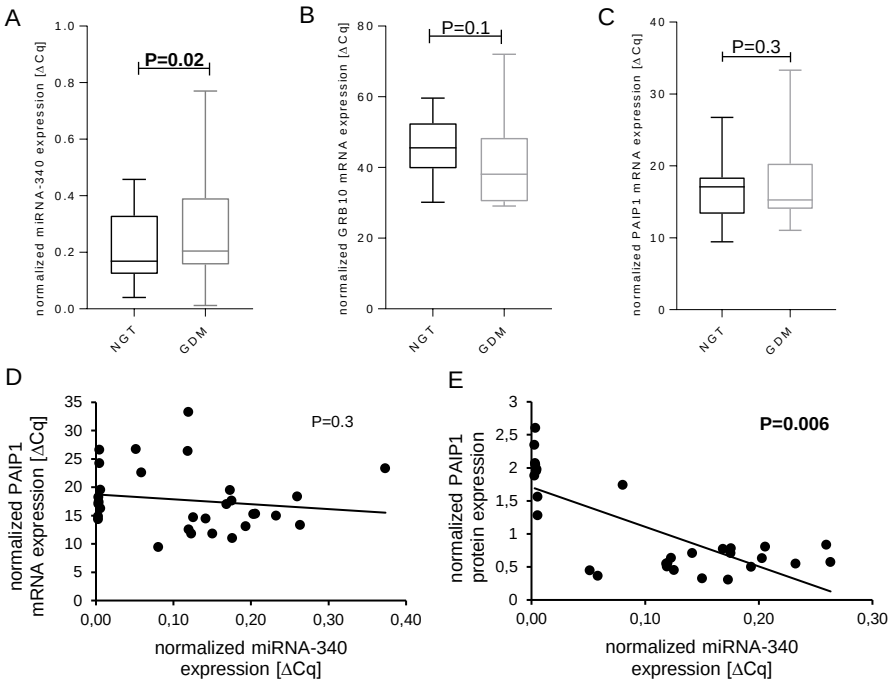

Figure 15: Expression of miRNA-340, GRB10 and PAIP1 in lymphocytes. RNA from lymphocytes of fifteen NGT (N=15) and GDM (N=15) women each was collected. Samples were paired based on maternal age and body mass index. Expression of (A) *miRNA-340* and both targets (B) *GRB10* and (C) *PAIP1* was compared.*miRNA-340* expression was correlated with PAIP1 (D) mRNA and (E) protein expression in lymphocytes. Shown are means ± SD. Statistically significant P-values (P≤0.05) are marked by bold fonts. Figure adopted from [1]

Assessment of *miRNA-340* in WBC from cord blood samples

As currently no indicator for GDM-associated fetal programming is available, we tested whether miRNA expression differences seen in WBC of GDM women during pregnancy are reflected in their offspring. Cord blood of children born from NGT (mNGT N=8) and GDM (mGDM N=8) pregnancies was collected. Quantification of *miRNA-340* followed by a paired analysis (paired based on fetal sex and birth size) no significant association of *miRNA-340* expression with maternal GDM was observed (P=0.2, supplemental Figure 6, S. 73). Anthropometric data of the children is shown in Table 3, S.32).

Regulation of *miRNA-340* and its target mRNAs by glucose and insulin in human lymphocytes

To identify which GDM-associated metabolic stimuli may contribute to the elevated *miRNA-340* in WBC and lymphocytes from GDM women, primary cell culture was conducted. Primary lymphocytes of pregnant women (N=8) were cultured in medium containing 5.5mM or 25.5mM glucose. These two glucose concentrations reflect fasting and postprandial glucose levels, respectively. Increased glucose levels were related with significantly reduced levels of *miRNA-340* (P<0.05, Figure 16A). Furthermore, influences of elevated insulin concentrations on lymphocyte *miRNA-340* expression were investigated. Cells were cultured in medium containing either 100nM insulin and 5.5mM glucose or 10nM insulin and 25.5mM glucose. The first condition should mimic increased fasting insulinemia and IR, the second

condition impaired insulin secretion in a severely diabetic pregnant woman. Again, clinical characteristics of the women donating the lymphocytes are shown in the Table 3, S.32).

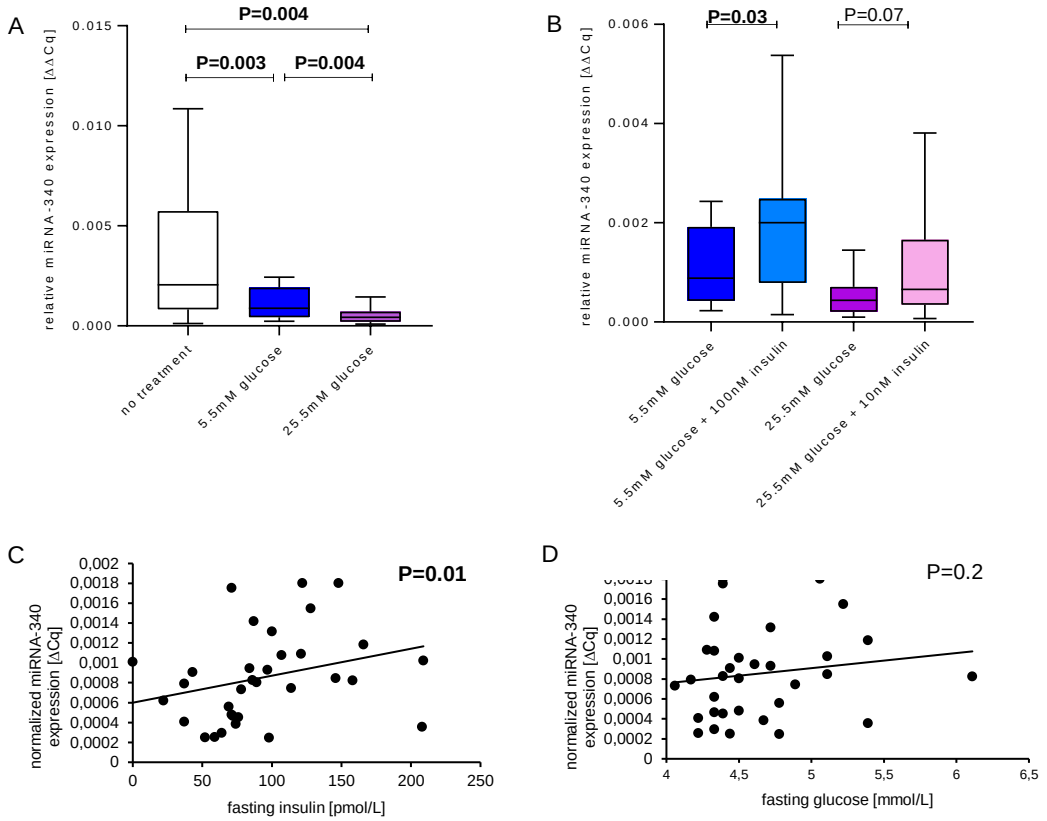

Figure 16: Expression of miRNA-340 in lymphocytes and whole blood cells in response to glucose and insulin. miRNA expression in non-cultured and cultured lymphocytes of pregnant women was investigated (N=8). (A) Comparison of non-cultured (no treatment, non-colored bars) and lymphocytes cultured with 5.5mM (blue bars) or 25.5mM (violet bars) glucose. (B) Comparison of cells cultured in medium containing 5.5mM glucose without and with 100nM insulin (light blue bars), respectively, and cells cultured in medium containing 25.5mM glucose without and with 10nM insulin (light violet bars), respectively. miRNA expression in whole blood cells (WBC) in relation to fasting (C) insulin and (D) glucose levels within the validation cohort (N=30). Shown are means ± SD. Statistically significant P-values (P≤0.05) are marked by bold fonts. Figure adopted from [1]

An increased *miRNA-340* expression was observed after stimulation of cells with 5.5mM glucose and 100nM insulin in comparison to cells stimulated with 5.5mM only (P=0.03; Figure 16B). Additionally, stimulation of cells with conditions mimicking impaired insulin secretion (higher glucose (25.5mM) and lower insulin (10nM)) was associated with a statistical trend for insulin-dependently elevated *miRNA-340* expression (P=0.07, Figure 16B). No differential miRNA expression of lymphocytes collected from NGT and GDM women was seen by MANOVA (P≥0.05) (data not shown).

To investigate if the *in-vitro* effects of insulin and glucose are reflected *in vivo*, a linear regression analysis of maternal fasting insulin and glucose levels with WBC *miRNA-340* expression was conducted. Maternal fasting insulin levels were positively associated with WBC *miRNA-340* expression (P=0.01, Figure 16C). No significant association was present between *miRNA-340* and fasting glucose (P=0.2, Figure 16D).

Lymphocytes of non-pregnant women being stimulated with identical metabolic stimuli did not show similar differences found in lymphocytes of pregnant women. Only stimulation with

10nM insulin at high glucose levels was associated with an increased expression of *miRNA-340* when compared to stimulation with high glucose only (P=0.05, Figure 17A and 17B).

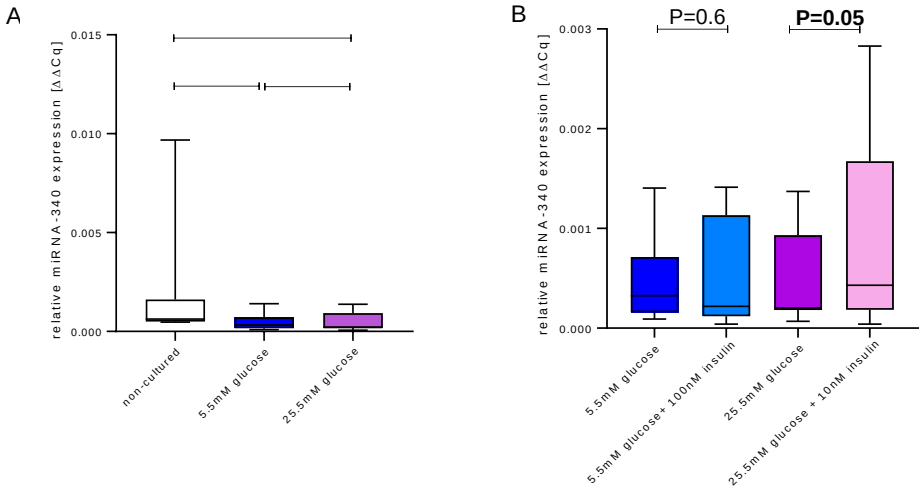

Figure 17: Expression of miRNA-340 in lymphocytes of non-pregnant women stimulated with glucose and insulin. miRNA expression in non-cultured and cultured lymphocytes of non-pregnant women was investigated (N=10). (A) Comparison of non-cultured (no treatment, non-colored bars) and lymphocytes cultured with 5.5mM (blue bars) or 25.5mM (violet bars) glucose. (B) Comparison of cells cultured in medium containing 5.5mM glucose without and with 100nM insulin (light blue bars), respectively, and cells cultured in medium containing 25.5mM glucose without and with 10nM insulin (light violet bars), respectively. Shown are means ± SD. Differences considered as statistically significant (P≤0.05) are marked by bold fonts. Figure adopted from [1]

miRNA and mRNA expression patterns in WBC related to maternal BMI

Secondary outcome variable was maternal BMI, as this parameter is an important risk factor for the development of GDM during pregnancy [116]. For detections of any associations between BMI and RNA expression independently of glucose tolerance status, again a non-paired analysis was conducted with the dataset collected from the screening group. For this secondary analysis the confounding covariates were: GDM, maternal age, pregnancy week, and maternal weight gain. We found four miRNAs to be positively associated with maternal BMI (P<0.05; FDR<0.1, Table 6). No overlap of this set of miRNAs with them found in GDM was observed. Furthermore, BMI-related differences in miRNA expression were small, as indicated by the low FC values. No down-regulated miRNAs were found. Additionally, no expression differences of piRNA and tRNA were detected. Analysis of the mRNA dataset within the secondary endpoint BMI revealed 25 mRNAs to be positively and 15 mRNAs to be negatively associated with maternal BMI (P<0.05; FDR<0.1, supplemental Table 4, S.78).

Table 6: MiRNAs associated with maternal BMI in whole blood cells of the screening group. Table 6 shows miRNAs significantly (P<0.05; false discovery rate (FDR)<0.1) associated with maternal BMI in the screening group. An unpaired analysis was conducted and adjusted for gestational diabetes, age, maternal weight gain, and pregnancy week. miRNAs are sorted by fold changes (FCs), shown in the 2nd column. Logarithmically transformed counts per million (logCPM), indicating the relative abundance of the transcript are shown in the 3rd column of the table. P-values and FDR are indicated in the 4th and 5th column, respectively.

Name	FC	P	FDR
miRNA-4473	1.075	3.8E-06	0.001
miRNA-199a-5p	1.074	1.3E-05	0.002
miRNA-339-5p	1.068	5.8E-05	0.006
miRNA-3653-5p	1.060	1.3E-03	0.096

Plasma miRNA pattern in pregnant NGT and GDM women

Cell-free plasma miRNAs are not only potential indicators of epigenetic differences, they are also proposed to have functional relevance in disease progression [112-114]. The main research question of the second part of Project lll was to identify GDM-associated miRNA in maternal plasma collected in gestational week 24-30.

As indicated in Figure 12 (S.31), all participants included in WBC miRNA pattern analysis were included in plasma miRNA pattern analysis. Therefore, anthropometric and metabolic characteristics of the participants (GDM N=9, NGT N=9) shown in Table 4 (S. 33) are similar to those shown in Table 3 (screening group, S.32). miRNA isolation from maternal plasma was followed by genome-wide miRNA-sequencing (conducted by Peter Huypens). Following the recommendation of Richa Batra, Institute of Computational Biology, Helmholtz Center Munich, the responsible bioinformatician of this subproject, an unpaired analysis was applied (confounding covariates: maternal age, BMI, weight gain and gestational week at time of blood collection). As indicated in supplemental Table 5 (S.79), 84 miRNA were shown to be negatively associated with maternal GDM. Supplemental Table 6 (S. 81) shows 52 miRNAs positively associated with maternal GDM. Comparisons between GDM-related miRNAs of maternal WBC and maternal plasma, revealed *let-7c-5p* to be the only overlapping miRNA positively associated with GDM.

Discussion

The increasing incidence of adiposity and T2D is discussed to be also a consequence of adverse fetal programming during pregnancy [6]. *In-utero* development of fetal IR in women with GDM [71-73] was proposed to be causative for the later development of T2D [117]. Underlying mechanisms are still unknown. An improved understanding might serve for the future development of prevention strategies.

Maternal GDM is associated with metabolic alterations (elevated plasma glucose, insulin and NEFA concentrations) which may alter placental function and subsequently fetal metabolism. GDM-associated metabolic alterations may also imply epigenetic differences leading to adverse fetal programming [6] and elevated maternal risk to develop T2D after a pregnancy accompanied by GDM [48, 102].

Project I: BMI-independent effects of maternal GDM on placental lipoinflammation

The placenta is an easily accessible mainly fetal tissue at the interface between maternal and fetal metabolism. It is of high relevance to gain insight into fetal metabolism [44] due to its metabolic and humoral control functions in pregnancy [45]. Elevated placental expression of genes involved in lipid metabolism [43, 118-120] and inflammation [119, 121-123] in obese pregnant women were proposed to be relevant for obesity-related fetal programming. To investigate if BMI-independent but GDM-associated differences of the placenta may contribute to adverse GDM-associated fetal outcome, such as IR [71, 72]; we analyzed maternal plasma and placental lipid profiles and their involvement on placental lipoinflammation and fetal metabolism (Figure 18).

In details, we observed significant elevation of SFA in maternal plasma of GDM women (Figure 18,①). Earlier findings in patients with T2D associated elevated plasma SFA with development of central and peripheral IR [64, 65, 106, 117, 124]. In pregnancies accompanied by GDM both maternal and fetal metabolism show elevated IR [71, 72]. Findings of elevated maternal plasma NEFA propose, that GDM-associated differences of maternal lipid metabolism could have relevance for development of elevated IR [35]. Our results of Project I propose that differences of maternal plasma lipid content and pattern may affect placental lipid metabolism: Placental lipases hydrolyze maternal lipoproteins. NEFA, derived from lipoproteins and from adipose tissue lipolysis, are taken up by fatty acid transport proteins of syncytiotrophoblasts [44]. We found elevated *FABP3* mRNA expression in placental tissue of GDM women, proposing an elevated NEFA uptake by syncytiotrophoblasts (Figure 18,②). Within this placenta-specific cell type, NEFA can become reesterified and finally stored as TG within lipid droplets. Earlier publications showed a positive association between maternal BMI and *PLIN2* expression as well as placental TG

content [43]. However, BMI-independent effects of GDM on placental TG were unknown, but of high relevance as epidemiological effects of GDM on fetal health indicate [6, 28]. Within our study we could show a 50-%-elevation of total placental TG content by GDM independently of BMI. Corresponding to that, we could show that placental tissue and trophoblasts of GDM women express higher levels of the lipid droplet associated protein *PLIN2* on mRNA expression level. Therefore, we conclude, that maternal GDM increases placental TG lipid storage independently of maternal BMI (Figure 18,③).

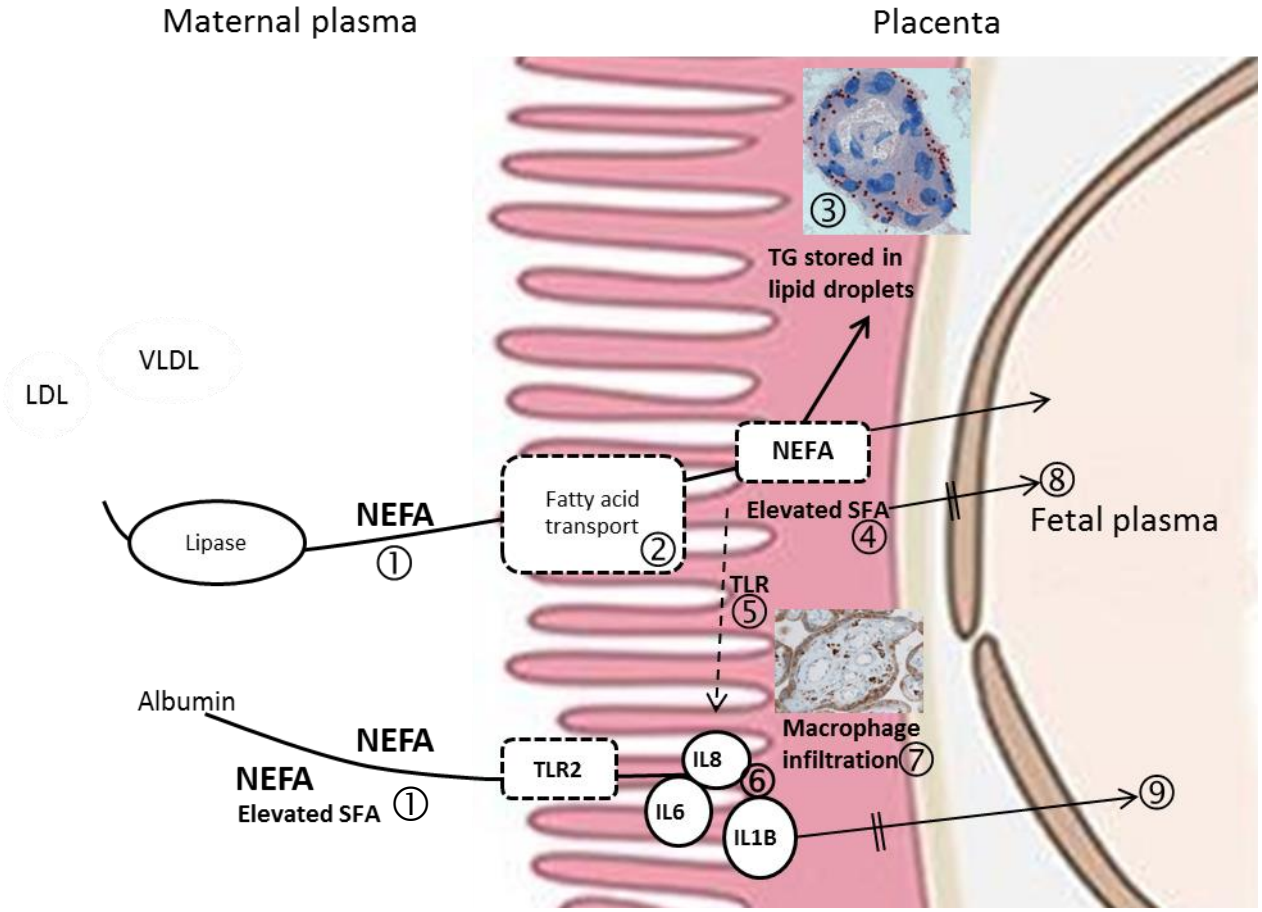

Figure 18: Placental lipoinflammation in women with GDM. This figure summarizes new findings of Project I. Details can be found in the text. Shortly, elevated saturated fatty acids (SFA) were quantified in maternal plasma and placental tissue①④. However, they are not transmitted to the fetus⑧. GDM-associated elevated amounts of TG are presumably stored in PLIN2-positive lipid droplets within the syncytiotrophoblast (PLIN2 staining indicated in red; picture friendly provided by B. Hirschmugl) ③. Presumably TLR-mediated elevated inflammatory gene expression ⑤⑥ is associated with elevated placental macrophage infiltration⑦, is however also not transmitted to the fetus⑨ (CD68 staining for placental macrophages is indicated in brown). All genes indicated showed an elevated expression in placental tissue or primary trophoblasts of GDM women.

Additionally to elevated total placental TG content, we could show in Project I for the first time that non-esterified SFA are elevated in placental tissue of GDM women (Figure 18,④). SFA, such as palmitate or stearate, were shown to contribute to the activation of TLR signaling *in vitro* and *in vivo,* earlier [125]. Similar to the classical ligand lipopolysaccharide, SFA can bind to different members of the TLR receptor family, such as *TLR2* and *TLR4* [104, 125, 126]. SFA-mediated activation of TLR receptors leads to MyD88-dependent activation of NF_KB, initiation of transcription of inflammatory genes and finally elevated cellular release of cytokines (IL6, IL1B, TNF-α) and chemokines (IL8) [127]. In this study, *TLR2* expression was

elevated in placental tissue of GDM women and positively associated with maternal plasma NEFA concentrations (Figure 18,⑤). Other mechanisms which may contribute to placental SFA signaling could be free fatty acid receptor (FFAR)- [128] or peroxisome proliferator-activated receptor (PPAR)-associated [129]. However they were not included in this study. Literature provides evidence that signaling via *TLR*, *FFAR*, and *PPAR* occurs in placental tissue [129, 130] and trophoblasts [100, 119, 131]. Maternal obesity was associated earlier with increased placental expression of inflammatory cytokines and chemokines [23, 131-133]. Significant elevations of, i.e., *IL1B* (FC=1.87) mRNA expression were found in placental tissue of obese compared to non-obese women [133]. Within Project I, we could identify elevated expression of proinflammatory cytokines (IL6 and IL1B) in whole placental tissue of GDM women, independently of maternal BMI (Figure 18,⑥). In comparison to obesity-associated placental effects on inflammatory gene expression [133], GDM-associated effects (independently of maternal BMI) have similar effect sizes. By correlation of maternal plasma NEFA concentrations with placental inflammatory gene expression, we could directly link maternal lipid metabolism with placental lipoinflammation (IL1B, IL6).

Furthermore, elevated placental expression of inflammatory genes was reflected by elevated inflammatory gene expression and protein release of primary trophoblasts isolated from GDM compared to NGT women. These findings could result from *in-vivo* effects of fatty acids on gene expression that are maintained during placental preparation and subsequent isolation and culture of the cells. To directly associate placental SFA content with inflammatory gene expression involved in the development of IR, we stimulated primary trophoblasts with SFA. Stimulation with palmitate, but not oleate, was associated with elevated mRNA and a statistical trend for elevated protein expression of IL6 and IL8. This result supports earlier findings indicating that SFA, contribute to the activation of *TLR* and nuclear factor kappa-light-chain-enhancer of activated B cells (NF_KB) [110, 126, 134]. SFA-induced elevated levels of proinflammatory cytokines are proposed to impair insulin sensitivity by reducing insulin receptor signaling [135, 136].

Additionally, SFA-mediated activation of TLR was shown to contribute to an increased gene expression of the chemokine *IL8* and, therefore, may be a potential mediator of placental macrophage infiltration [137] (Figure 18,⑥). Comparisons of villous macrophage content between NGT and GDM women revealed an elevated amount of CD68-positive macrophages in placental tissue of GDM women (Figure 18,⑦). GDM-associated attraction and accumulation of placental macrophages could enhance placental lipoinflammation [123].

Based on these results, we hypothesized that GDM-associated disturbance of maternal and placental lipid metabolism might be the missing link to the *in-utero* development of fetal IR present in GDM [30, 44, 71, 72]. To investigate this hypothesis, we focused on the potential effect of GDM-associated differences of SFA and inflammatory

parameters in fetal cord blood. Based on our findings in placenta of GDM women, we assumed to find elevated concentrations of SFA (Figure 18,⑧) and proinflammatory parameters (Figure 18,⑨) in fetal cord blood of GDM offspring. However, we could not find GDM-associated elevations. Major SFA species were not elevated in fetal cord blood of GDM offspring. Rather, a statistical trend for reduced concentration of sum of SFA was seen in the cord blood of GDM offspring. Additionally several cytokines were found to have a lower concentration in offspring of GDM women. Therefore, we conclude, that GDM-associated placental lipoinflammation and accumulation of SFA are not directly transmitted to the fetal circulation and are, therefore, unlikely to be involved with the *in-utero* development of adverse fetal outcome such as elevated risk to develop T2D later in life. Altogether, the findings of elevated SFA in placental tissue of GDM women, but not in the cord blood of GDM offspring propose a potential protective role of the placenta for the developing fetus at least in well controlled GDM pregnancies at the end of pregnancy. Future studies need to investigate if GDM-associated differences of the metabolomic profile of the placenta and the cord blood are present. Furthermore, epigenetic studies should be performed to investigate mechanisms for basal mRNA expression differences found in trophoblasts of NGT and GDM women.

We acknowledge limitations of Project I (partly also valid for Project II). We did not investigate epigenetic effects which are proposed to be a possible mechanism contributing to adverse fetal programming [138, 139]. NEFA can act as epigenetic regulators. Short fatty acids can inhibit histone deacetylase activity [140]. Additionally, stimulation of skeletal muscle cultures with palmitate led to elevated methylation of peroxisome proliferator-activated receptor gamma coactivator 1-α (*PGC-1α*), a central regulator of energy metabolism [138]. Future studies need to be conducted to investigate the nutrient-dependent epigenetic regulation of genes involved in placental metabolism and fetal development. Furthermore, our findings may not reflect the placental lipid profile and gene expression as a whole, as we collected placental samples from the fetal facing surface of the placenta, only. Also, influences of dietary differences between NGT and GDM women on the data were not taken into account, as no dietary information during pregnancy was collected in the pregnancy study. Moreover, limited sample sizes available in defined sub-experiments are a further limitation of this study; however replication of findings in these subgroups supports robustness of the data.

In summary, based on our findings, we could show that maternal GDM exerts BMI-independent effects including elevation of maternal plasma and placental SFA concentrations as well as fatty acid-mediated lipoinflammation. However, neither inflammatory parameters nor SFA appear to be transmitted to the fetus. Therefore, we

conclude that placental lipoinflammation of GDM mothers is not directly involved in GDM-associated adverse fetal programming.

Project II: BMI-independent effects of maternal GDM on placental apelinergic system

As neither elevated amounts of SFA nor inflammatory parameters were seen in cord blood of newborns from GDM women, other mechanisms modifying fetal risk of IR *in utero* and, subsequently, future adiposity and T2D [6] need to be explored. Beside of classical pregnancy hormones [39, 75, 76], the placenta releases hormones involved in regulation of metabolism, such as APL. GDM-associated differences in the expression of the placental apelinergic system could contribute to metabolic *in-utero* programming with life-long consequences of the offspring [83, 141].

Within this study, we could show elevated *APL* mRNA expression in placental tissue of GDM women compared to NGT controls. Furthermore, we could proof that trophoblasts are the cell type responsible for placental *APL* expression. Corresponding to elevated placental mRNA expression in GDM, primary trophoblasts collected from GDM women were shown to release higher amounts of *APL in vitro*. Within our study, the amount of cultured cells was too low for trophoblast *APL* mRNA or protein quantification. However, earlier publications propose that insulin [142, 143] as well as proinflammatory parameters [144, 145] and polyunsaturated NEFA [146] could stimulate APL release (i.e., in adipocytes). Therefore, the impact of other GDM-associated metabolic and inflammatory stimuli on trophoblast *APL* release should be investigated in future studies. Furthermore, as *APL* expression is proposed to be epigenetically regulated, studies on *APL*-targeting miRNAs and APL DNA methylation pattern [147] should be conducted.

To investigate if GDM-associated differences in placental trophoblast APL release have any effect on APL cord blood concentration, fetal APL concentrations were quantified in female and male NGT and GDM offspring. No significant differences were observed in the overall cohort. However, as sex-specific differences of plasma APL levels were shown, we investigated GDM-associated effects in male and female offspring, separately [111]. We found a statistical trend for elevated fetal APL concentrations in female offspring of GDM women. Subtly elevated *in-utero* APL concentrations may in long-term shape fetal metabolism [77] also via targeting central pathways [79]. Future studies should focus on quantification of APL in well characterized larger cohorts. Fetal sex should be considered as important confounder in such studies [111].

Additionally to systemic effects, trophoblast APL release may have autocrine effects via targeting the trophoblast *APJ* receptor. Differential placental APJ expression was shown to have a high relevance for fetal health [83, 148, 149]. Within this study, we could show an elevated *APJ* expression in placenta and trophoblasts of GDM women. Via

immunohistochemical staining it became visible, that trophoblasts are the major placental cell type of APJ expression. Stimulation of primary trophoblasts with the SFA palmitate was associated with elevated *APJ* mRNA expression. The stimulatory effect was more pronounced in trophoblasts isolated from GDM women, an observation that may in parts also result from GDM-associated epigenetic differences. As SFA are shown to be more abundant in placental tissue of GDM women, the positive correlation between placental palmitate concentration and *APJ* mRNA expression underlines the *in-vitro* results. Further differences in response to stimulation were absent although earlier findings indicate that hyperinsulinaemia could elevate *APJ* expression in adipose tissue of humans [150]. Placental angiogenesis and sufficient placental vascularization, both co-regulated by *APJ*-signaling, were shown to be essential for placental function such as transplacental nutrient transfer [82]. Reduced *APJ* signaling in preeclampsia [148] and *in-utero* growth restriction [151] was shown to have a negative impact on placental angiogenesis. In contrast to that, GDM was shown to be associated with elevated placental vascularization [152, 153] that may also result from SFA-mediated increased *APJ* expression. Further studies should investigate the underestimated role of *APJ* for placental development and fetal programming [154].

In summary, we could show an elevation of placental and trophoblast *APL* and *APJ* expression. Furthermore, palmitate was shown to increase *APJ* mRNA expression in human primary trophoblast. Currently, it is not clear whether elevated GDM-associated placental APL release contributes to fetal cord blood APL concentrations.

In conclusion, we propose that GDM-associated differences of *APJ* expression could be of high relevance for placental vascularization [155] and therefore fetal development. Furthermore, the autocrine effects of altered GDM-associated APL release could give insights in adverse metabolic programming of GDM offspring [5].

Project III: GDM-associated effects on maternal whole blood miRNA patterns

The biological relevance of miRNAs in the development of obesity and diabetes has been accepted in the last years [92-94]. Several studies have been conducted to associate miRNA expression patterns with the development, progression, and prevention of diabetes. Additionally to circulating plasma miRNAs [95, 156], transcriptomic differences in easily accessible blood cells are of growing relevance [97]. We conducted this study to see if alterations in miRNA-dependent programming related to GDM can be detected in maternal whole blood.

Sequencing of miRNA isolated from WBC of NGT and GDM women during pregnancy revealed an elevated expression of 29 miRNAs in WBC of GDM women. To investigate the relevance of these sequencing results, we conducted qPCR validation experiments in a

larger validation group. In this experiment, out of six selected miRNAs, *miRNA-340* was the only miRNA which we could validate to be elevated in lymphocytes of GDM women. Additionally, quantification of *miRNA-340* expression was conducted in isolated lymphocytes. Again, significantly elevated *miRNA-340* expression was found in cells collected from GDM women. *miRNA-340* is a miRNA newly described in the context of GDM or diabetes in general. Only one earlier study described elevated circulating levels of *miRNA-340* in newly diagnosed T1D children [157]. For three more miRNA (*miRNA-142, miRNA-143, miRNA-let-7g*), which showed a statistical trend for elevated expression in WBC of GDM women, more literature findings are present: elevated circulating concentrations of *miRNA-142* were seen in obese and non-obese T2D patient [158]. Additionally, metformin therapy reduced circulating concentrations of *miRNA-142* in these patients. *miRNA-143* was shown to have an increases expression in rat peripheral mononuclear blood cells [159]. Moreover, in liver of diabetic mouse model, increased *miRNA-143* expression was detected. Additionally, *miRNA-143* overexpression was associated with impaired insulin sensitivity and glucose homeostasis [160]. In our WBC screening group, seven members of the *miRNA-let-7* family were positively related with GDM in WBC in miRNA sequencing. The *miRNA-let-7* family is shown to regulate peripheral glucose metabolism [161]. Additionally, whole-body transgenic mice overexpressing *let-7a, -7d,* or *7f* were shown to be glucose-intolerant [162]. Similarly to our finding, diabetes or diabetes-associated metabolic stimuli were associated with increased expression of members of the *miRNA-let-7* family, *in vivo* and *in vitro* [163, 164] .

To understand if elevated *miRNA-340* expression levels have any biological relevance, the expression of target genes was analyzed. For *PAIP1*, significant GDM-related reduction in mRNA expression could be found in maternal WBC. Additionally, by applying multiple regression analysis, negative correlation between *miRNA-340* and PAIP1 protein expression was observed in lymphocytes. The role of *PAIP1* specifically in blood cells is currently not understood. However, *PAIP1* was described as an initiator of protein translation [165]. *miRNA-340-dependent* reduced PAIP1, present in our cohort, may control an adequate protein synthesis in blood cells.

For identification of GDM-related metabolic stimuli contributing to elevated *miRNA-340* expression cell culture with primary lymphocytes was conducted within our study. A significant positive insulin effect on the expression of *miRNA-340* was observed. The *in-vitro* findings are reflected by a positive correlation of maternal fasting insulin levels with *miRNA-340* expression *in vivo*. Primary cell culture conducted from non-pregnant women led to no marked expression differences upon insulin treatment. This result indicates that pregnancy-associated factors may be permissive for this *miRNA-340* expression inducing effect of insulin.

Finally, we investigated if WBC miRNA-340 expression could be a potential future indicator for adverse fetal programming of GDM offspring. Increased insulin and glucose levels in cord blood samples of children born from GDM pregnancies were reported [40, 166] and may lead to epigenetic differences in the fetal metabolism. However, we could not find any significant elevation of *miRNA-340* in fetal WBC of newborns of GDM mothers. This finding proposes that metabolic stimuli contributing to *miRNA-340* expression are different in mother and offspring.

Additionally to the genome-wide sequencing of maternal WBC, miRNA pattern of maternal plasma of NGT and GDM women was conducted. Subjects who were included in the WBC sequencing group, donated plasma at the same time. Circulating miRNAs are discussed to originate from apoptotic cells from tissues with a high cellular turn over [167]. In the context of GDM, these tissues might be adipose tissue, liver, pancreas and/or placenta [112, 113, 168]. Furthermore, WBC are proposed to contribute to plasma miRNA content [167, 169]. In contrast to cancer-related plasma miRNAs [170], future studies need to investigate the predictive value of plasma miRNA in metabolic diseases such as diabetes. Although the origin of the 84 plasma miRNA negatively and the 52 plasma miRNA positively associated with maternal GDM is ultimately not clear, GDM associated differences of circulating miRNAs give insights in maternal disease progression [113] and also fetal programming [114]. Defined miRNAs are cell-type-specific. Differential plasma patterns may give insights in tissue-specific influences of GDM. Within the plasma miRNA dataset, we found both splicing variants of *miRNA-199* to be more abundant in pregnant GDM women. Literature proposes these miRNAs to be tissue-specific indicators of pancreas [171] and liver [172, 173] dysfunction in diabetes. *miRNA-92*, also found to be more abundant in pregnant GDM women, was shown to be elevated in the placenta and plasma of pregnant women of macrosomic offspring [174]. MiRNAs are proposed to be taken up and released by the placenta. Therefore, maternally elevated abundance of *miRNA-92* may give insights in fetal health or even fetal adverse programming.

Finally, we found an elevated abundance of three members of the miRNA-let-7 family (*let-7c, -d,* and *–f*) in plasma of GDM women. As mentioned above, expression of this family is shown to respond to elevated glucose levels [162], also in WBC of GDM women (*let-7c-5p*). Currently, the effects of maternally dysregulated plasma miRNAs on the placenta and the fetus are not understood. However, it is theoretically conceivable that GDM-associated elevation of miRNA-let-7 family members in the fetus of GDM women may contribute to the development of IR [162].

We acknowledge limitations of the study. The effect of fatty acids on miRNA expression was not investigated. As differences of the lipid metabolism are of high relevance, this should be considered in future studies. Additionally, the putative reversibility of miRNA

derangement should be assessed in GDM follow-up investigations. Furthermore, regarding the findings of GDM-associated plasma miRNA, qPCR validation should be conducted in a bigger validation cohort. Moreover, all candidates found to be associated with GDM in maternal whole blood and plasma should be studied in placenta and fetal whole blood.

In summary, our results provide evidence of miRNA-dependent programming of WBC related to GDM. The functional studies indicate that the GDM-related *miRNA-340* responds to insulin and glucose in cultured lymphocytes and may therefore be of importance in hyperinsulinemia-induced differences in gene expression. *PAIP1*, a predicted target mRNA of *miRNA-340*, shows reduced mRNA expression related in maternal GDM. Furthermore, we could show, that GDM is associated with differences in the pattern of maternal cell-free miRNAs.

In conclusion, we found potential future indicators of GDM based on WBC and plasma miRNA profiling. Future prospective studies focusing on the predictive value these indicators need to evaluate applicability.

Methods

Participants and maternal and fetal blood collection

From an ongoing pregnancy study, pregnant NGT and GDM women were selected and blood was collected during pregnancy (pregnancy week 27±2, Project I and II NGT N=18; GDM N=18, Project III screening: NGT N=8 and GDM N=8, validation: NGT N=30 and GDM N=30, plasma miRNA NGT N=9, GDM N=9). To compare findings in pregnant compared to non-pregnant women, blood was collected for lymphocyte isolation from the Tuebingen Family (TUEF) study [175]. For determination of the fetal cord-blood plasma lipid profile (Project I) samples of the pregnancy cohort were used (NGT N=11, GDM N=7). For quantification of fetal inflammatory parameters samples from the PEACHES cohort (NGT N=52, GDM N=52) [176] were available. Fetal cord blood collection was conducted at birth. Plasma was aliquoted immediately after blood collection. Samples were stored at -80°C. The pregnancy study and the PEACHES study were both approved by the local Ethic Boards and were performed in accordance with the ethical standards laid down in the Helsinki Declaration. Written informed consent was obtained from all study participants. Except of 16 women of the PEACHES cohort, none of the women underwent insulin treatment. Characterization of participants was conducted by the physicians and clinical scientists of the Clinical Study Center University Hospital Tübingen, Regina Ensenauer and Sarah Perschbacher (Ludwig Maximillians University Munich and Heinrich Heine University Düsseldorf), and the group of Gernot Desoye (Medical University Graz).

Oral glucose tolerance test and clinical chemical analyses

The participants underwent a 5-point 75-g OGTT after overnight fasting. Venous blood samples were obtained at 0, 30, 60, 90, and 120min for determination of plasma glucose using a bedside glucose analyzer (YSI, Yellow Springs, OH, USA). GDM was diagnosed according to the IADPSG recommendations for the diagnosis and classification of hyperglycemia in pregnancy [55]. The PEACHES participants either had a 75-g OGTT or a 50-g GCT (N=30) followed by a 75-g OGTT in case of a positive result (1-h glucose ≥7.8 mmol/L) [177]. In Project III, whole-body ISI was calculated for a defined group of participants. Therefore glucose and insulin values during the 5-point OGTT were collected and ISI was calculated as proposed by Matsuda and DeFronzo [178]: $10{,}000/[c(Glc_0)*c(Ins_0)*c(Glc_{mean})*\ c(Ins_{mean})]^{½}$(with c=concentration, Glc=glucose and Ins=insulin). For Project I and II clinical data are shown in Table 1 (S.20). Study workflow and exclusion criteria are shown in supplemental Figure 1 (S.71). Clinical data and study workflow of Project III are shown in Table 3 and 4 (S.32), and Figure 12 (S.31).

Sampling of placental tissue and cord blood as well as characteristics of participants

At delivery, placental tissue was collected strictly following a high-quality random sampling protocol [179, 180] (tissue with poor blood supply in proximity of the umbilical cord) and

extensively washed (4-10°C, 140mmol/l NaCl solution, for 30 seconds) immediately (NGT N=19; GDM N=11). Thereafter, tissue from the surface facing the fetal circulation was prepared. Tissue for lipid and protein analysis underwent another preparation (definition of quantity, other washing procedure, addition of proteinase and phosphatase inhibitors (complete and phosstop, Roche, Basel, Switzerland)) and was association with a reduction of available samples. Tissue was stored at -80°C until further analysis of RNA, proteins and lipids.

Determination of the fatty acid profile of different lipid classes in placenta and maternal as well as fetal cord blood

Maternal (NGT N=18; GDM N=18) and fetal cord blood plasma (NGT N=11; GDM N=7) as well as placental samples (NGT N=8, GDM N=8) were collected and processed for determination of lipid profiles by strictly following pre-analytical recommendations described earlier [181]. Placental samples were homogenized in PBS containing 1% Triton X-100 (Sigma-Aldrich, Taufkirchen, Germany) with a Tissue Lyser (Qiagen, Hilden, Germany). Placental extracts and plasma samples were separated into five lipid subfractions, i.e., cholesteryl esters (CE), diacylglycerols (DG), NEFA, phospholipids (PL), and triacylglycerols (TG), using thin layer chromatography. Methodological details of thin layer chromatography and further determination of lipid profiles by gas chromatography were described earlier [182]. In total, 18 FAs were quantified (C14:0; C16:0, C16:1-n7, C18:0, C18:1-n7, C18:1-n9, C18:2-n6, C18:4-n3, C20:0, C20:3-n6, C20:4-n6, C22:0, C22:4-n6, C22:5-n3, C22:5-n6, C22:6-n3, C24:0, C24:1-n9). Experiments were conducted by Marketa Kovařova.

Cell culture experiments

In Project I and II, primary trophoblasts were isolated from placentas of NGT and GDM women selected for similar maternal BMI (NGT N=7; GDM N=7). Isolation and quality control (human choriongonadotropin (β-hCG) release, cytokeratin 7 (CK7) (marker for syncytiotrophoblasts), vimentin (marker for fibroblasts), and histocompatibility antigen, class I, G–staining (widely expressed, however not in syncytiotrophoblasts) was conducted as described previously [183] [43]. We observed no reduction in β-hCG levels and only positive staining of the trophoblast-specific protein CK7 (supplemental Figure 7, S.73). After isolation, cells were kept in DMEM medium (Lonza, Basel, Switzerland) for 48h followed by a 24h stimulation with medium, 100µM palmitate or 100µM oleate (Sigma-Aldrich, Taufkirchen, Germany) coupled to 10% bovine serum albumin solution (BSA) (Sigma-Aldrich, Taufkirchen, Germany) and corresponding controls for 24h [109]. Cell culture supernatants were collected after 24-h of stimulation. In Project III, human lymphocytes were isolated from heparinized venous blood samples during pregnancy week 24-32. Briefly, the heparinized blood was diluted with PBS (Lonza) in the ratio 1:1 and layered on Biocoll Separating Solution® (Biochrom). Afterwards, the sample was subjected to centrifugation (500g for 20min). The white layer representing the lymphocytes was aspirated gently and transferred

aseptically to new tubes. The cell suspension was washed and cultured in sterile DMEM medium (GIBCO) with different glucose (5.5mM or 25mM) and insulin (Insuman Rapid, Sanofi) (10nM) concentrations. The medium was supplemented with 10% fetal bovine serum (Biochrom) and 1% penicillin-streptomycin (Lonza). The number of cells was adjusted to 0.5×10^6 cells/well in 12-well plates. After 20-h incubation at 37°C, the cells were harvested.

Total RNA isolation

For Project I and II, RNA was isolated from homogenized placental tissue (N=30) and trophoblasts (N=14) using miRNeasy (Qiagen). For Project III, whole blood was collected in PAXgene Blood RNA Tubes (PreanalytiX) after overnight fasting. Total RNA was isolated using the Paxgene Blood miRNA kit (PreanalytiX) according to the manufacturer's specifications. From freshly isolated and 20-h stimulated primary lymphocytes, total RNA was isolated with miRNeasy micro kit (Qiagen). The quality of the obtained WBC's RNA was subsequently evaluated on the Agilent 2100 Bioanalyzer System. Samples with a RIN value ≥ 8.0 were used for downstream library construction.

miRNA isolation from maternal plasma

During pregnancy week 27±2, maternal blood was collected after overnight fasting. Maternal plasma was stored till isolation of miRNAs at -80°C. For miRNA isolation, miRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany) was used with following adaptations proven to increase amounts of miRNA isolated: 400µl of stored plasma was thawn and centrifuged at 14.000g for 10min at 4°C. Supernatant was mixed with 1.2ml TRI Reagent LS *(*Sigma-Aldrich, St. Louis, USA), gently vortexed for 10sec followed by 10min of incubation at room temperature (RT). As glycogen is proposed to be a carrier of miRNA, 1.2µl of a 10mg/ml glycogen solution were added to each sample. After addition of 320µl chloroform, samples were again vortexed for 45sec and incubated for 5min at RT. Finally, following the manufactures' recommendations for the rest of the procedure, samples were centrifuged at 14.000g for 20min at 4°C. miRNA was eluated two times with 15µl water.

cDNA synthesis and quantitative PCR (qPCR)

mRNA expression of *CRY2, FABP3, FABP4, FAT, GRB10, ID2, IL1B, IL6, IL8, LIPG, LPL, PAIP1, PITPNB, PLIN2, SCARB, SPRY3, TNF-α, TLR2, TLR4* and the house keeping gene *RPS13* was determined on the Light Cycler 450 (Roche) as shown earlier [184]. For mRNA qPCR, 2.5ng of cDNA was used. *RPS13* was shown to be stably expressed in trophoblasts of NGT and GDM women and not influenced by maternal BMI (data not shown). $\Delta\Delta C_q$ values are shown when mRNA expression of treated cells is given relative to a control condition, i.e., mRNA expression of untreated cells. ΔC_q values are shown to compare basal mRNA expression, normalized to a housekeeping gene. Reverse transcription of miRNA was conducted as recommended by the manufacturer. Briefly, 500ng total RNA was reversely transcribed with 5x miScript HiFlex Buffer (miScript II RT kit, Qiagen) under following conditions: 37°C for 60min, 95°C for 15min, immediately on ice. This was followed by qPCR

(miScript SYBR Green PCR kit, Qiagen). QPCR measurements of both miRNAs and mRNAs were conducted in duplicates. For miRNA qPCR, 10ng cDNA were used. Expression of *miRNA-19a-3p* (MIMAT0000073), *miRNA-19b-3p* (MIMAT0000074), *miRNA-142-5p* (MIMAT0000433), *miRNA-143-3p* (MIMAT0000435), *let-7g-5p* (MIMAT0000414), *miRNA-340-5p* (MIMAT0000750), and the house keeping gene U6 small nuclear 6, (*RNU6B*) was determined on a LightCycler 450 (Roche) in following conditions: activation 95°C for 15min, quantification (45 cycles) 94°C for 15sec, 55°C for 30sec, 70°C for 30sec. In order to control for primer specificity, a melting curve was performed. Primer sequences and fluorescent probes are shown in supplemental Table 7 (S. 82).

Library preparation and RNA sequencing

NEBNext small RNA library Prep Set for Illumina and NuGen Ovation RNA-Seq system v2 were used according to the manufacturer's recommendations to generate small and long RNA-Seq libraries. For long RNA-Seq, the obtained cDNAs were sheared with a Covaris Focused-ultrasonicator prior to adaptor ligation. Finally, libraries were sequenced on an Illumina HiSeq2500 platform by Peter Huypens.

Cytokine, chemokine, and APL quantification from placental tissue, cell culture supernatant and fetal sera

Placental tissue pieces were extensively washed with cold PBS and recommended concentrations of proteinase and phosphatase inhibitors (complete and phosstop, Roche, Basel, Switzerland) and stored at -80°C till further processing (NGT N=6; GDM N=6). Tissue pieces (50mg) were homogenized by Qiashredder (Qiagen, Hilden, Germany) and ultrasound in protein lysis buffer (10nM Tris containing complete and phosstop, pH 7.4). Total protein concentrations were determined with Pierce BCA Protein Assay Kit (Thermofisher, Waltham, USA). Placental IL1B, IL6 and IL8 protein levels were quantified using Quantikine HS ELISA (R&D, Minneapolis, USA). For IL1B and IL8 undiluted protein lysates and for IL6 protein lysates diluted 1:10 were used. Quantification was conducted in duplicates and protein concentrations were normalized for total cellular protein. Cytokine and chemokine concentrations in undiluted primary trophoblast cell culture supernatants were quantified using Quantikine HS ELISA (R&D, Minneapolis, USA, NGT N=7; GDM N=7). Inflammatory cytokines in fetal cord blood sera were quantified using Bio-Plex Pro Human Cytokine-27-plex Kit (BioRad Laboratories, Biorad, Hercules, USA) following the manufacturers' recommendations (diluted 1:4). Each pair selected based on maternal BMI (NGT N=52; GDM N=52) was run on one plate to avoid inter-assay variance. Concentrations outside the standard curve were not considered for statistical analysis leading to reduced sample sizes: Granulocytecolony stimulating factor (G-CSF) (N=56), GM-CSF (N=53), IL6 (N=57), and IL15 (N=65), interferone (IFN)-γ (N=71), IL8 (N=98), MIP-1α (N=96), regulated on activation (RANTES) (N=58), and vascular endothelial growth factor (VEGF) (N=67). Quantification of APL in cell culture supernatant and fetal sera was conducted with an ELISA

(Phoenix Pharmaceuticals, CA, USA). Following the recommendations of the manufacturer sera were diluted 1:4. Cell culture supernatants were not diluted.

Protein visualization and quantification by Western blot

For Project l and ll, placental tissue was shredded and homogenized in a protein lysis buffer (50mM Hepes, pH 7.0), 150mM NaCl, 1.5mM $MgCl_2$, 0.1mM EGTA, 10% glycerol, 1% Triton x-100) (Roth, Karlsruhe, Germany). The proteins from tissue and cell lysates (50µg) were resolved on a 7.5% or 10% Tris-HCl gel and blotted onto nitrocellulose membranes. For Project lll, primary lymphocytes were isolated as described above and washed in PBS (Lonza, Basel, Switzerland). Protein isolation was conducted with AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Hilden, Germany) and protein was solved in Lämmli buffer containing 62.5mM Tris, pH 6.8), 2% SDS, 10% Glycin, and 3% beta-mercaptoethanol. Protein concentrations were determined with Qubit Protein Assay Kit (Thermo Fisher Scientific, Dreieich, Germany). Unspecific binding sites were blocked with 5% dry milk powder in TBS-Tween prior to overnight incubation with primary monoclonal TLR2 (1:500, Abcam, Cambridge, England) or monoclonal APJ (1µg/ml, Merck-Millipore, Darmstadt, Germany) or polyclonal GRB10 and monoclonal PAIP1 (1:1000 Abcam, Cambridge, UK, both) antibody. GAPDH (1:1000; Sigma Aldrich, St. Louis, USA) was used as loading control. The incubation with primary antibody was followed by 1-h incubation with a HRP-coupled secondary antibody (1:3000). Antibodies were diluted in 5% dry milk powder in TBS-Tween. Protein bands were detected and quantified with Chemidoc touch Image lab software (Biorad, Hercules, USA).

Quantification of macrophages

Paraffin-embedded, serial sections of placental tissue (NGT N=3; GDM N=3, selected based on fetal sex and maternal BMI) were incubated with a primary monoclonal antibody against CD68 (1:3000, clone KP-1, Dako, Jena, Germany) (brown staining). The primary antibody was detected using the Opti-View system (Roche-Ventana, Multimer Technology, Basel, Switzerland). Haematoxylin-eosin staining was used as counterstaining. Immune cell infiltration of the villous stroma was quantified by counting CD68-positive villi in a blinded manner. In more detail, intermediate villi similar in size and area from morphological comparable sections of placental tissue slides were selected. From each placenta, four pictures from three slides were used for quantification. Percentage of CD68-positive placental villi related to the total amount of selected villi was calculated.

Statistical analysis

Project l and ll

Data are presented as individual data, means +/- SD, or means +/- SEM as indicated. Multiple linear regression analysis was conducted for all *ex-vivo* experiments. We used maternal BMI, gestational age, fetal sex as confounding covariates, and GDM vs NGT as independent variable. To avoid overadjustment, small subgroups of BMI- and fetal sex-

matched samples (CD68-quantification) were analyzed with adjustment for gestational age, only. The descriptive clinical data presented in Table 3 and 4 (S.32) were analyzed by two-tailed Student's t-test. The data from *in-vitro* cell culture experiments were analyzed by one-tailed Student's t-test (two-group comparisons) or analysis of variance (ANOVA) with Dunnett's post-hoc test (three- and more-group comparisons). Differences with P≤0.05 were considered statistically significant.

Project lll

For RNA sequencing, raw read counts for miRNAs and mRNAs were generated by overlapping the positions of the mapped reads with annotations obtained from miRbase (doi: 10.1093/nar/gkt1114) and RefSeq, (doi: 10.1093/bioinformatics/btp616) respectively. Statistical analysis of the read counts can be found at the NCBI Gene Expression Omnibus (GEO record: GSE92772) and were conducted using the edgeR (doi: 10.1093/bioinformatics/btp616) package for R. For the identification of GDM-related differential expression of sncRNAs, lncRNAs, and mRNAs, in the screening group and validation group, a paired analysis (two-tailed Student's t-test) was conducted after adjustment for pregnancy week and maternal weight gain within the first half of the pregnancy. For this paired analysis, P-values<0.05 and FDR<0.1 were considered as significant. For visualization of the results of the screening group, an interaction network analysis was performed. Based on miRNA-mRNA *in silico* target site predictions obtained from Target Scan (version 6.2) (doi: 10.1016/j.cell.2004.12.035), the previously described miRlastic R approach for the identification of potential regulatory interactions between significantly regulated miRNAs and mRNAs by incorporating their expression profiles was applied [185]. Briefly, elastic net regression was used for performing feature selection on a set sequence-based target predictions to identify inversely regulated miRNA-target relationships. For qPCR validation experiments within the validation and cord blood group, paired two-tailed Student's t-tests were conducted (JMP, SAS, Cary, USA). In cases where the direction of expected differences was defined by earlier experiments one-tailed paired Student's t-tests were conducted (JMP, SAS, Cary, USA). Using univariate linear regression analysis, WBC miRNA-340 expression was correlated with maternal fasting glucose and insulin levels. Applying multiple linear regression, lymphocyte miRNA-340 expression was correlated with PAIP1 mRNA and protein expression after correction for two batches of sample preparation (JMP, SAS, Cary, USA). To this end the batch was introduced as dummy variable in the multiple linear regression models. In order to investigate effects of maternal BMI, an unpaired analysis of the results from the screening group was conducted, by multiple linear regression analysis. Adjustments for this secondary analysis were maternal GDM, maternal age, weight gain, and pregnancy week. All computational analysis of the WBC subproject, were conducted by Steffen Sass.

For analysis of differential GDM-related abundance of plasma miRNAs, we followed the recommendation of the bioinformatician Richa Batra conducting the multiple regression analysis adjusting for maternal age, BMI, weight gain, fetal sex, and gestational age at time of blood collection (pregnancy week 27±2).

Contributions

Following co-workers supported the present work:

Project I and II:

- Recruitment and clinical characterization of study participants: Andreas Fritsche, Louise Fritsche, Corinna Sailer, Vera Schmid, Department of Internal Medicine, Division of Endocrinology, Diabetology, Angiology, Nephrology and Clinical Chemistry, University Hospital Tübingen (Clinical study centre)
- Maternal and fetal as well as placental lipid pattern: Markéta Kovářová, Erwin Schleicher, Andreas Peter, Department of Internal Medicine, Division of Endocrinology, Diabetology, Angiology, Nephrology and Clinical Chemistry, University Hospital Tübingen
- Isolation of placental trophoblasts: Renate Michlmaier, Christian Wadsack, Gernot Desoye, Department of Obstetrics and Gynaecology, Medical University Graz
- Collection of fetal plasma serum and clinical characterization of the PEACHES cohort: Sarah Perschbacher and Regina Ensenauer, Institute for Social Pediatrics and Adolescent Medicine, Ludwig-Maximilians-Universität München and Division of Experimental Pediatrics and Metabolism, University Children's Hospital, Heinrich Heine University.

Project III:

- RNA sequencing: Peter Huypens, Insitute of Experimental Genetics, Helmholtz Center Munich
- Biostatistical analysis: Steffen Sass, Richa Batra, Institute of Computational Biology, Helmholtz Center Munich

Acknowledgments

This doctoral dissertation arose in the Institute for Diabetes Research and Metabolic Diseases of the Helmholtz Center Munich at the University of Tübingen. The project was co-chaired by Prof. Harald Staiger and Prof. Andreas Fritsche under the supervision of Prof. Dr. Dr. Hans-Ulrich Häring. I thank them all for the interest and support for my work.

I acknowledge the various technical contributions to my work that led to achieve the current results. Placenta collection and preparation during night and day would have not been possible without Alke, Carina, Karin, Lisa, Melanie, and especially Roman. Kubrom conducted and introduced me to immunohistochemical staining of placental tissue. Ulrike and Birgit conducted many qPCRs for this work. Without the patient support of Léonie, reimplementation of Western blot in the lab would not have been possible. Elisabeth collected primary lymphocytes for my primary cell culture experiments and, with the great support of Alke, isolation and quantification of plasma miRNAs became real. I enjoyed the discussions and technical challenges we faced. The personal interaction within different subgroups of the institute enriched my work experience. We learned a lot about this new research topic. I would also like to thank for advices, support and friendly cooperation with Anja, András, Felicia, Siggi, Christoph, Madhura, Marketa and Estela. During my time here I could collect experiences, but also friends!

Finally, I acknowledge the support of our collaborations partners in Munich, Düsseldorf and Graz. Special thanks to Gernot Desoye and Christian Wadsack.

This project was partially funded by grants of the DZD, EASD and DDG (Allgemeine Projektförderung 2015 and 2017). Those grants allowed me to participate and present my scientific data at several congresses, summer schools, and meetings.

Wege geht man nicht alleine.
Ich danke Allen die mich auf diesem Weg unterstützt haben,
Kompromisse eingegangen sind und Geduld bewiesen haben.

Zusammenfassung

Projekt I und II der vorliegenden Arbeit beschäftigen sich mit Auswirkungen von Gestationsdiabetes (GDM) auf die zukünftige Gesundheit des Kindes. Epidemiologische Studien weisen darauf hin, dass mütterlicher GDM das spätere Risiko für Übergewicht und Diabetes beim Kind erhöht. Weitere Ergebnisse weisen darauf hin, dass Feten von GDM-Müttern bereits *in utero* eine Insulinsresistenz (IR) des Gehirns entwickeln, was ursächlich für die spätere Entwicklung von Typ-2-Diabetes sein könnte.

Ziel dieser Projekte war es plazentale und fetale Veränderungen in Abhängigkeit von mütterlichem GDM zu identifizieren. Zunächst wurden Lipidprofile des mütterlichen Blutes und der Plazenta von normal glucosetoleranten (NGT) und GDM-Frauen erstellt. Dabei konnte gezeigt werden, dass sich erhöhte Mengen an gesättigten Fettsäuren (ges. FS; z.B.: Palmitat) aus dem mütterlichen Blut in plazentalem Gewebe wiederfinden. Ebenfalls konnte gezeigt werden, dass GDM mit einer erhöhten plazentalen Expression von *PLIN2*, inflammatorischen Genen (*TLR2, IL1B,* und *IL6*) und Apelin (*APL*) sowie seinem Rezeptor *APJ* einhergeht. Der Vergleich der Expression dieser Gene in primären Trophoblasten isoliert aus NGT und GDM-Frauen spiegelt die plazentalen Befunde wieder. Zudem führt die Stimulation von primären Trophoblasten mit Palmitat zu einer erhöhten Expression von *PLIN2* und der inflammatorischen Gene sowie *APJ*. Außerdem ist GDM mit einer erhöhten Einwanderung von Makrophagen in die Plazenta assoziiert. Dies könnte auf eine gesteigerte *IL8*-Expression zurückzuführen sein. Abschließend wurde untersucht ob sich GDM-assoziierte Veränderungen der Plazenta im fetalen Blut wiederfinden und damit zur intrauterinen Programmierung beitragen könnten. Dabei wurden weder signifikant erhöhte Mengen an ges. FS, noch erhöhte Konzentrationen von Zytokinen oder *APL* im Nabelschnurblut von Neugeborenen der GDM-Mütter gefunden. Dies deutet darauf hin, dass andere Mechanismen an der Entwicklung der intrauterinen Insulinresistenz beteiligt sind.

Neben direkten metabolischen Einflüssen auf die Plazenta und den fetalen Metabolismus wurde untersucht ob es im Blut von Schwangeren mit GDM zu epigenetischen Veränderungen kommt. Zunächst wurde das miRNA-Expressionsprofil von Blutzellen aus NGT und GDM-Frauen verglichen. In mütterlichen Blutzellen konnte dabei eine erhöhte Expression von *miRNA-340* identifiziert werden. *In-vitro*-Ergebnisse zeigen, dass Insulin die Expression dieser miRNA steigert. Auch *in vivo* gibt es eine positive Korrelation der Plasmainsulinwerte mit der Expression von *miRNA-340* in Blutzellen. Zusätzlich wurde der Einfluss dieser dysregulierten miRNA auf mögliche Zielgene untersucht. Dabei konnte eine negative Korrelation zwischen der *miRNA-340* Expression und des PAIP1-Proteins in mütterlichen Lymphocyten gezeigt werden. In mütterlichem Plasma konnten ebenfalls GDM-assoziierte miRNA-Expressionsmuster gefunden werden. Folgestudien sollten nun den prädiktiven Wert dieser miRNAs als mögliche neue Marker für GDM untersuchen.

Summary

Project 1 and 2 focus on the influence of maternal gestational diabetes (GDM) on fetal future health. Epidemiological data indicate that offspring of pregnancies accompanied by maternal GDM have elevated risk of developing overweight and type 2 diabetes (T2D) later in life. Recent results propose that fetal insulin resistance (IR) exists already *in utero* and could be causative for development of overweight and T2D later.

Scientific goal of these projects was to investigate placental GDM-associated alterations contributing to fetal programming. Maternal plasma and placental lipid profiles were prepared. These profiles indicate that elevated amounts of saturated non-esterified fatty acids (SFA, i.e. palmitate) of the maternal circulation can be found in placental tissue and contribute to an elevated placental gene expression of *PLIN2* and inflammatory genes (*TLR2, IL1B*, and *IL6*). Gene expression of these genes in primary trophoblasts isolated from NGT and GDM women resembles expression in whole placental tissue. Furthermore, stimulation of primary trophoblasts with palmitate leads to increased gene expression of *PLIN2*, inflammatory genes, apelin (*APL*) and it's receptor *APJ*. Additionally, GDM was shown to be associated with an elevated placental macrophage accumulation. This finding may result from elevated SFA-mediated *IL8* expression in placenta of GDM women. Finally, we investigated if GDM-associated alterations of placental lipid profiles and gene expression contribute to differences of cord blood lipid and inflammatory cytokine concentrations or differences in cord blood APL concentrations. As no GDM-associated elevated fetal plasma SFA and inflammatory cytokine concentrations or differences of APL concentration were found we propose that other mechanisms could contribute to GDM-associated development of *in-utero* IR.

In conclusion, our results indicate that GDM-associated alterations do not directly influence fetal metabolism. *In-utero* development of fetal IR may therefore result from other mechanisms.

Additionally to direct metabolic influences of GDM on the placenta and the fetal metabolism, we investigated if epigenetic differences in maternal blood are associated with GDM. Therefore, the miRNA expression profile of maternal blood was compared between NGT and GDM women. An elevated expression of *miRNA-340* could be found in maternal whole blood cells (WBC). *In-vitro* results of this project indicate that insulin increases the expression of this miRNA. Furthermore, a positive correlation between maternal plasma insulin and WBC *miRNA-340* is observed. Additionally the influence of the differentially expressed miRNA on poteintial target genes was investigated. A negative correlation between *miRNA-340* expression and PAIP1 protein expression in lymphocytes was seen. In maternal cell-free plasma, further GDM-associated miRNA expression patterns are present. Future studies should investigate the predictive value of these new GDM-associated indicators.

Publications

Parts of this PhD thesis are published in following publications:
Accepted:

- A computational biology approach of a genome-wide screen connected miRNAs to obesity and type 2 diabetes, Gottmann P, Ouni M, Saussenthaler S, Roos J, Stirm L, Jähnert M, Kamitz A, Hallahan N, Jonas W, Fritsche A, Haering H-U, Staiger H, Blüher M, Fischer-Posovszky P, Vogel H, Schuermann A. Molecular Metabolism, Available online 15 March 2018, ISSN 2212-8778,
- Maternal whole blood cell miRNA-340 is elevated in gestational diabetes and inversely regulated by glucose and insulin. Stirm L, Huypens P, Sass S, Batra R, Fritsche L, Brucker S, Abele H, Hennige AM, Theis F, Beckers J, Hrabě de Angelis M, Fritsche A, Häring HU, Staiger H.Sci Rep. 2018 Jan 22;8(1):1366.
- Early hypermethylation of hepatic Igfbp2 results in its reduced expression preceding fatty liver in mice. Kammel A, Saussenthaler S, Jähnert M, Jonas W, Stirm L, Hoeflich A, Staiger H, Fritsche A, Häring HU, Joost HG, Schürmann A, Schwenk RW. Hum Mol Genet. 2016 Jun 15;25(12):2588-2599. Epub 2016 Apr 28.

In submission:

- BMI-independent effects of gestational diabetes on human placenta; Stirm L, Kovářová M, Michlmaier R, Fritsche L, Siegel-Axel D, Schleicher E, Peter A, Pauluschke-Fröhlich J, Brucker S, Abele S, Wallwiener D, Perschbacher S, Preissl H, Wadsack C, Häring H-U, Ensenauer R, Desoye G, Fritsche A, Staiger H

In preparation:

- Unique sequence based (USB) small RNA profiling, Jeske T, Huypens P, Stirm L, Hoeckele S, Weigert C, Staiger H, Fritsche A, Beckers J, and Hastreiter M

Parts of this PhD thesis were presented at following conferences:

- 09/2017 Oral Presentation at the 53rd EASD annual meeting, Lisbon, Portugal
- 04/2017 Poster Presentation at the IR 2017, Nizza, France
- 04/2017 Oral presentation and Poster Presentation DZD Research School, Nizza, France
- 09/2016 Oral Presentation at the 52nd EASD annual meeting, Munich, Germany
- 09/2016 Poster presentation at the DZD Summer School, Munich, Germany
- 05/2016 Poster presentation at the 52nd annual Deutscher Diabeteskongress, Berlin, Germany
- 09/2015 Poster presentation at the DZD Summer School, Stockholm, Sweden
- 05/2015 Oral presentation at the15th DZD Workshop, Munich, Germany
- 05/2015 Poster presentation at the 51st annual Deutscher Diabeteskongress, Berlin, Germany

Subprojects of this PhD thesis were funded by grants I applied for:

- 03/2017 Grant: Allgemeine Projektförderung 2017 to L. S. (DDG) (10.000 €)
- 03/2015 Grant: Allgemeine Projektförderung 2015 to L.S. (DDG) (10.000 €)

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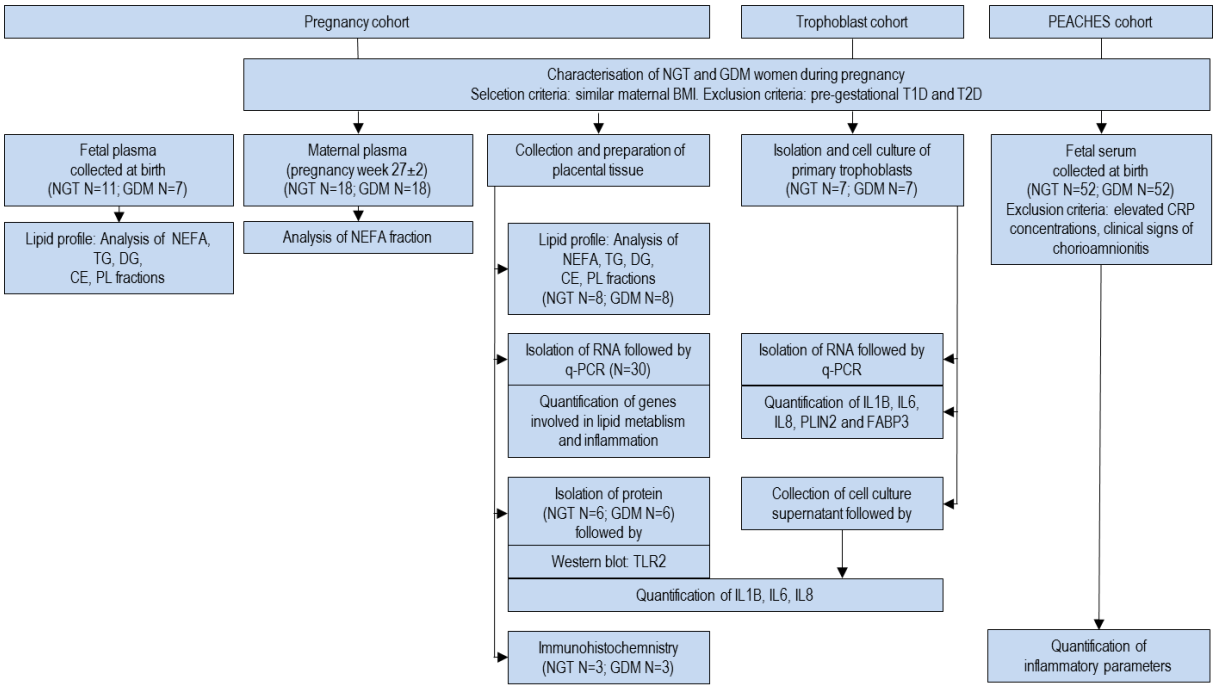
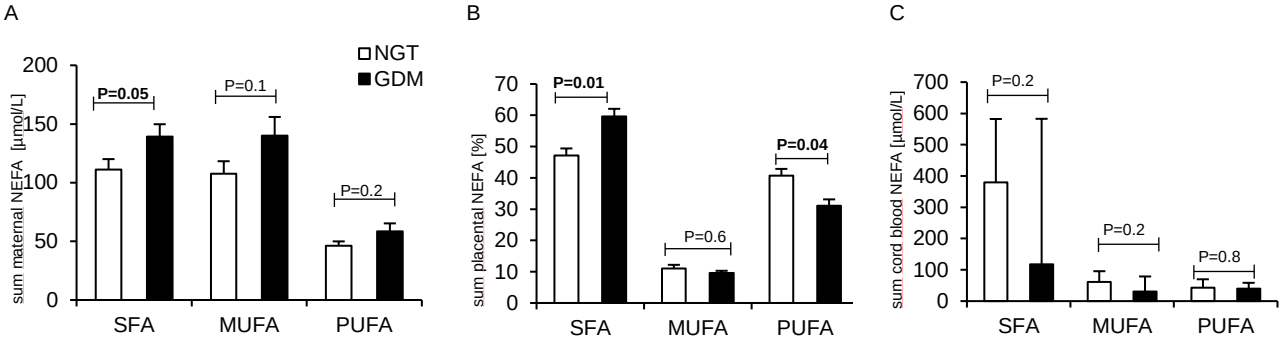
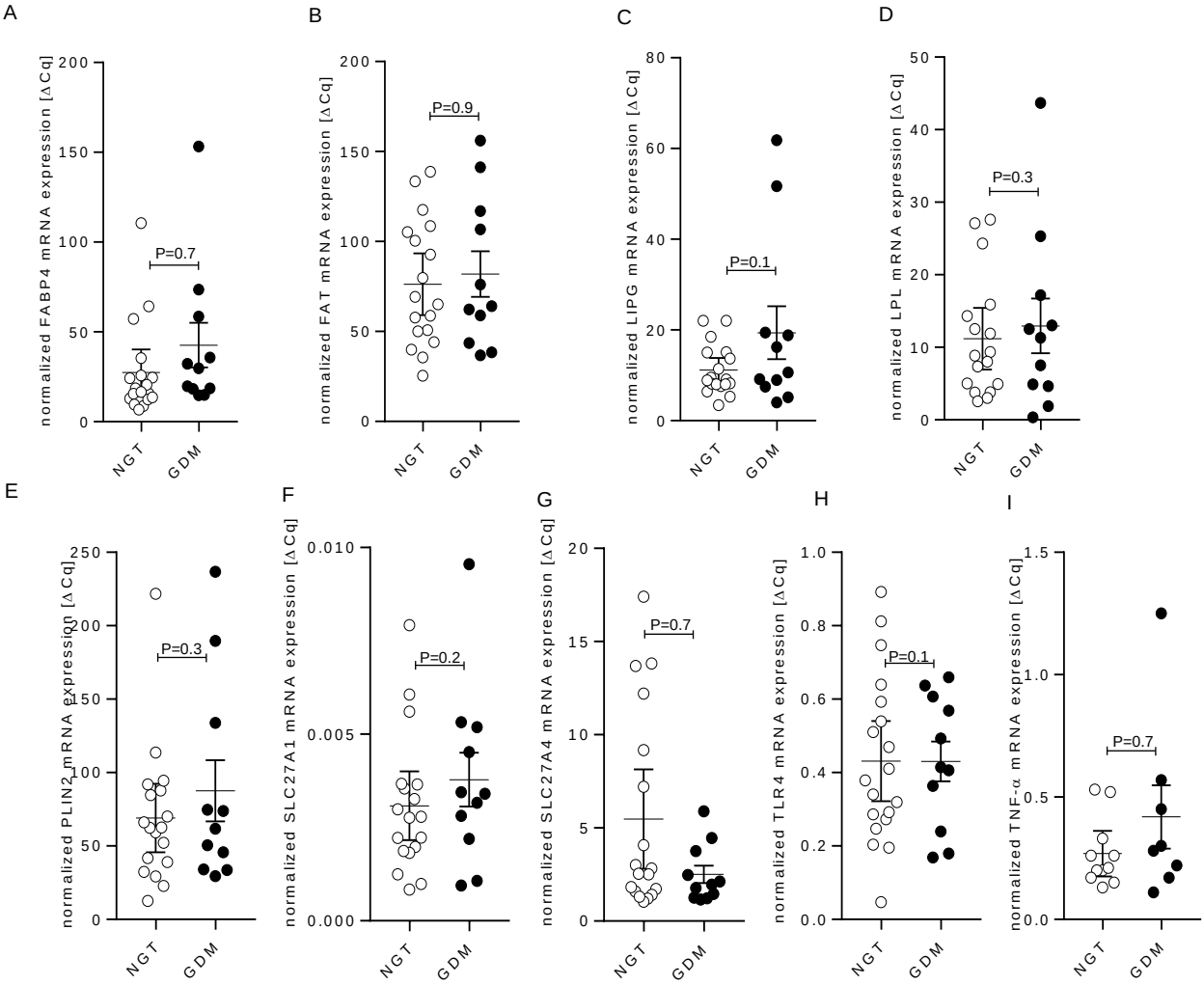
Appendix



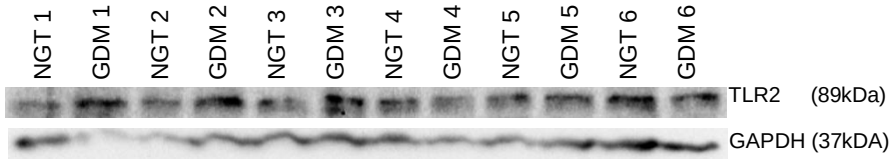
Supplemental Figure 1: Available samples and conducted experiments in each cohort are indicated.



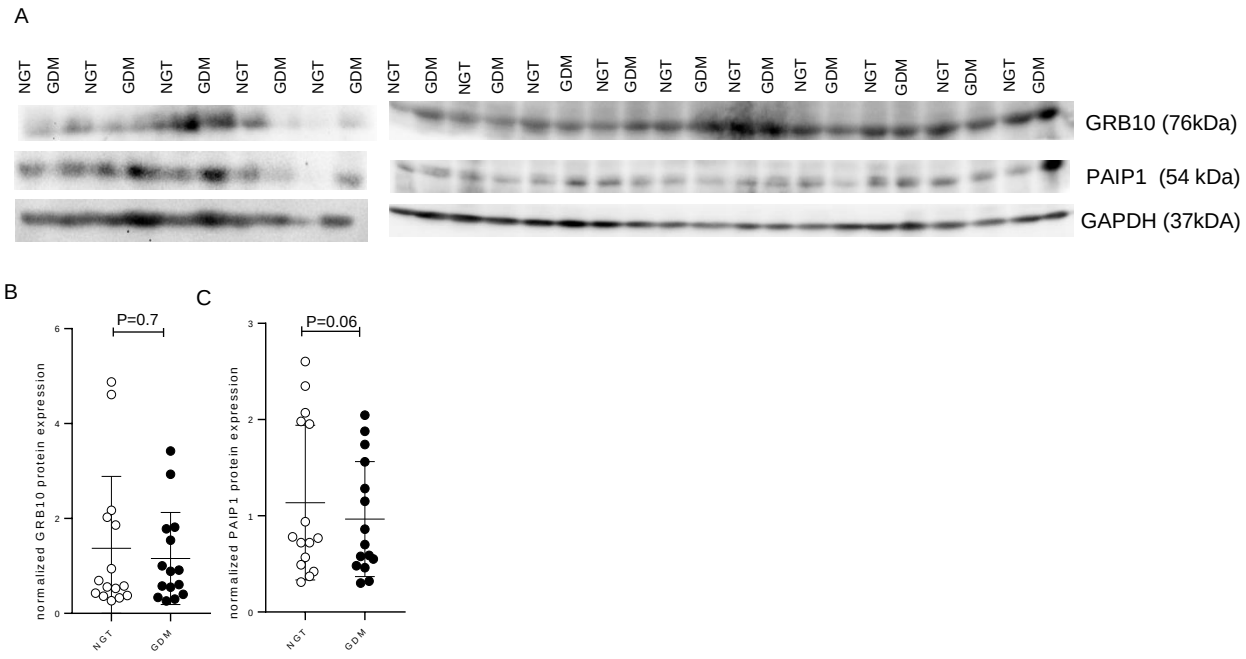
Supplemental Figure 2: Non-esterified fatty acids (NEFA) in mother, placenta and fetus. Saturated (SFA), mono-unsaturated (MUFA) and poly-unsaturated (PUFA) NEFA-profile in (A) maternal plasma (NGT N=18; GDM N=18), (B) placental tissue (NGT N=19; GDM N=11), and (C) fetal cord blood (NGT N=11; GDM N=7). Shown are means ± SEM. White columns show results of NGT and black columns of GDM women. For analysis of maternal NEFA profile fetal sex was not considered as confounder (confounding covariates: BMI, gestational age). Significant differences (P≤0.05) are marked with bold fonts.



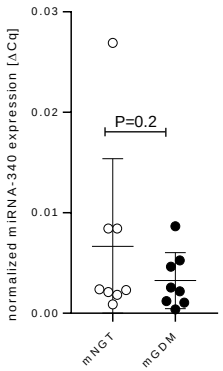
Supplemental Figure 3: Gene expression in whole placental tissue. (A-I) mRNA expression of *FABP4, FAT, LIPG, LPL, PLIN2 SLC27A1, SLC27A4, TLR4,* and *TNF-α* in placental tissue of normal glucose tolerant (NGT N≥9) and gestational diabetes (GDM N≥8) women



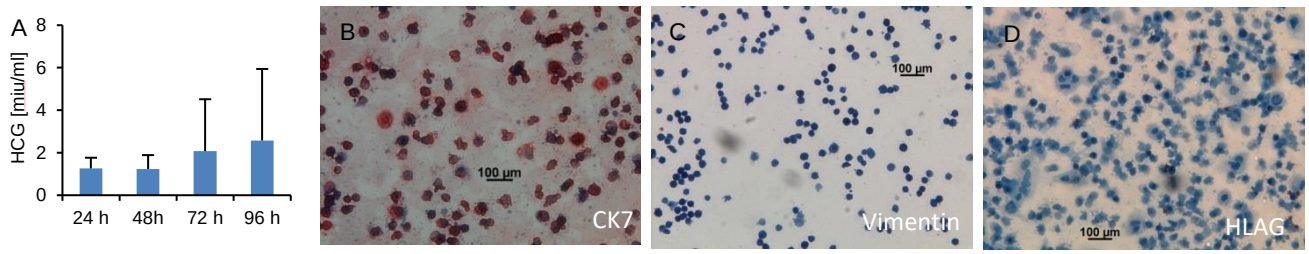
Supplemental Figure 4: Protein expression of TLR2 and GAPDH by Western blotting. For protein quantification, protein was isolated from placental tissue of normal glucose tolerant (NGT N=6) and gestational diabetes (GDM N=6) women. Samples were selected based on maternal body mass index.



Supplemental Figure 5: Protein expression of GRB10 and PAIP1 in lymphocytes. For protein quantification, protein was isolated from lymphocytes of normal glucose tolerant (NGT N=15) and gestational diabetes (GDM N=15) women. Samples were paired based on maternal age and body mass index. (A) Western blots with antibodies against GRB10 and PAIP and the reference gene GAPDH. Protein expression of (B) GRB10 and (C) PAIP1 after normalization with GAPDH. Shown are means ± SD.



Supplemental Figure 6: Expression of miRNA-340 in fetal cord blood WBC. *miRNA-340* expression in cord blood WBC of children each born from maternal NGT (mNGT) and maternal GDM (mGDM) (N=8, each) pregnancies was investigated. Samples were paired based on birth size and fetal sex. Shown are the means ± SD.



Supplemental Figure 7: Quality control of primary trophoblasts. (A) Human choriongonadotropin (β-hCG) values of primary trophoblasts (N=7) after 24h, 48h, 72h and 96h in cell culture supernatant. Immunocytochemical staining of primary trophoblasts with antibody against (B) cytokeratin 7 (CK7) a trophoblast-specific marker, (C) vimentin and (D) histocompatibility antigen, class I, G (HLAG). Red staining indicates positively marked cells

Supplemental Table 1: Lipid profile of placental tissue of NGT and GDM women. Placental lipid profile of normal glucose tolerant (NGT N=8) and gestational diabetes (GDM N=8) women was compared. Placental extracts were separated into five lipid subfractions, i.e., cholesterol ester (CE), diacylglycerol (DG), non-esterified fatty acid (NEFA), phospholipid (PL), and triacylglycerol (TG). All data are given as absolute numbers or means ± SD. Significant differences (P<0.05) are indicated in bold fonts.

	CE		DG		NEFA		PL		TG	
	NGT	GDM	NGT	GDM	NGT	GDM	NGT	GDM	NGT	GDM
C14:0 [%]	5.45±2.86	4.17±2.21	1.37±0.36	1.62±0.25	2.78±1.01	3.4±1.34	0.45±0.12	0.52±0.23	5.43±1.63	4.09±1.87
C16:0 [%]	26.46±8.71	25.37±5.41	28.58±1.45	29.15±2.69	**26.08±2.42**	**30.02±2.28**	19.09±1.02	20.51±3.26	33.15±3.03	31.06±2.81
C16:1-n7 [%]	1.67±0.93	1.02±0.35	1.70±1.07	1.55±1.02	0.43±0.11	0.46±0.08	0,19±0.09	0.26±0,10	1.37±0.36	1.46±0.11
C18:0 [%]	19.25±8.01	19.94±6.88	18.65±1.8	19.34±2.17	**17.57±3.81**	**25.28±4.82**	14.92±0.65	16.19±2.79	17.87±7.89	17.05±4.07
C18:1-n7-cis [%]	0.9±0.37	1.62±1.92	1.13±0.17	1.05±0.27	1.57±0.29	1.25±0.45	1.66±0.27	1.56±0.26	1.83±0.62	2.04±0.61
C18:1-n9 [%]	8.56±3.34	8.95±3.96	5.46±0.57	5.84±1.80	9.27±3.19	8.11±1.80	8.19±0.74	7.84±0.99	17.15±9.21	19.24±6.81
C18:2-n6 [%]	21.29±14.74	22.25±9.25	5.91±0.69	5.04±1.98	9.64±2.00	7.86±2.21	9.77±1.03	9.56±1.79	9.08±4.79	10.48±2.49
C18:4-n3 [%]	2.54±1.30	1.56±0.61	0.13±0.04	0.17±0.07	1.62±1.10	1.27±0.57	0.14±0.09	0.32±0.41	2.48±1.86	1.87±1.08
C20:0 [%]	0.49±0.22	0.48±0.19	0.5±0.09	0.52±0.17	0.34±0.16	0.45±0.08	**0.21±0.04**	**0.31±0.16**	0.47±0.23	0.40±0.25
C20:3-n6 [%]	7.07±2.37	7.31±2.43	7.49±2.84	7.07±3.02	5.91±2.02	5.11±1.69	**7.31±1.76**	**5.85±1.16**	3.93±2.94	3.87±1.41
C20:4-n6 [%]	4.93±4.43	5.06±3.03	27.73±.1.62	26.68±7.38	15.98±4.08	11.96±4.43	26.35±1.80	25.84±6.02	4.23±1.86	5.30±2.74
C22:0 [%]	0.31±0.14	0.28±0.10	**0.05±0.02**	**0.10±0.04**	0.22±0.09	0.20±0.13	0.71±0.12	0.88±0.29	0.29±0.14	0.27±0.11
C22:4-n6 [%]	0.62±0.19	0.59±0.31	0.69±0.12	0.57±0.24	3.07±1.78	1.66±1.49	2.10±0.52	2.00±0.31	0.93±0.52	0.80±0.25
C22:5-n3 [%]	0.23±0.09	0.29±0.15	0.10±0.05	0.15±0.06	0.24±0.07	0.21±0.10	0.41±0.08	0.41±0.11	0.30±0.14	0.30±0.12
C22:5-n6 [%]	0.44±0.19	0.49±0.14	0.31±0.07	0.40±0.16	0.71±0.17	0.70±0.25	0.97±0.26	0.86±0.25	0.48±0.32	0.45±0.20
C22:6-n3 [%]	0.75±0.15	0.87±0.35	1.55±0.51	1.85±0.76	3.34±1.60	2.02±0.75	6.10±1.29	5.28±1.32	1.49±0.99	2.06±1.19
C24:0 [%]	0.30±0.08	0.34±0.17	0.13±0.05	0.20±0.09	0.19±0.07	0.24±0.10	0.86±0.17	1.05±0.84	0.41±0.29	0.30±0.18
C24:1-n9 [%]	0.40±0.11	0.44±0.14	0.24±0.14	0.26±0.12	0.22±0.11	0.25±0.10	0.77±0.15	1.02±0.59	0.49±0.37	0.42±0.22
MUFA [%]	9.87±3.49	11.00±3.66	6.83±0.72	7.15±2.00	11.06±3.42	9.61±2.06	0.11±0.01	0.10±0.02	19.46±9.54	21.70±6.97
PUFA [%]	37.64±16.90	38.14±11.18	43.9±2.16	41.92±4.56	40.73±6.42	31.04±6.26	0.54±0.01	0.51±0.08	22.91±5.14	25.12±5.10
SFA [%]	51.77±19.24	50.08±13.52	49.27±2.43	50.93±4.39	47.18±6.71	59.60±7.45	0.36±0.01	0.39±0.07	57.63±12.73	53.18±8.18

Supplemental Table 2: mRNAs negatively associated with maternal GDM in whole blood cells of the screening group. Supplemental Table 2 shows miRNAs significantly (P<0.05; false discovery rate (FDR)<0.1) negative associated with GDM. A paired analysis was conducted and adjusted for maternal weight gain and pregnancy week. mRNAs are sorted by fold change (FC), shown in the 3rd column. Logarithmic counts per million (logCPM), indicating the relative abundance of the transcript, are shown in the 4th column of the table. P-values and false discovery rates (FDR) are indicated in the 5th and 6th column.

Symbol	Name	FC	logCPM	P	FDR
SFMBT2	Scm-like with four mbt domains 2	0.507	3.309	1.22E-03	0.095
FAIM3	Fas apoptotic inhibitory molecule 3	0.505	5.313	7.18E-04	0.072
LOC100272216	uncharacterized LOC100272216	0.500	5.703	1.06E-03	0.091
CTSW	cathepsin W	0.494	3.278	9.91E-04	0.090
LILRB1	leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 1	0.491	5.151	3.21E-04	0.057
CD163	CD163 molecule	0.491	2.798	1.31E-03	0.098
ZXDB	zinc finger, X-linked, duplicated B	0.481	2.388	1.15E-03	0.093
LOC441081	POM121 membrane glycoprotein (rat) pseudogene	0.479	7.214	3.96E-04	0.062
ZMAT1	zinc finger, matrin-type 1	0.478	2.947	6.28E-04	0.070
NLRP2	NLR family, pyrin domain containing 2	0.476	3.708	5.77E-04	0.070

LOC100288778	WAS protein family homolog 1 pseudogene	0.475	3.146	6.15E-04	0.070
HLA-DMB	major histocompatibility complex, class II, DM beta	0.473	4.998	2.55E-04	0.052
TAS2R40	taste receptor, type 2, member 40	0.473	2.630	1.07E-03	0.091
NUTM2G	NUT family member 2G	0.467	2.241	1.05E-03	0.091
TTI1	TELO2 interacting protein 1	0.466	2.294	1.00E-03	0.090
TERF1	telomeric repeat binding factor (NIMA-interacting) 1	0.464	2.368	7.99E-04	0.077
HLA-DMA	major histocompatibility complex, class II, DM alpha	0.462	5.675	1.27E-04	0.049
ATAT1	alpha tubulin acetyltransferase 1	0.455	3.310	2.12E-04	0.052
MOV10	Mov10 RISC complex RNA helicase	0.454	1.977	1.12E-03	0.093
ABCG1	ATP-binding cassette, sub-family G (WHITE), member 1	0.453	1.887	1.22E-03	0.095
ZBTB25	zinc finger and BTB domain containing 25	0.450	3.026	2.78E-04	0.052
GPRASP1	G protein-coupled receptor associated sorting protein 1	0.449	1.931	1.03E-03	0.091
ADGRL1	adhesion G protein-coupled receptor L1	0.448	2.225	5.59E-04	0.069
PRPF31	pre-mRNA processing factor 31	0.446	3.825	1.36E-04	0.049
TSPAN3	tetraspanin 3	0.446	2.770	2.36E-04	0.052
VSTM1	V-set and transmembrane domain containing 1	0.446	4.128	1.62E-04	0.052
TNFSF12	tumor necrosis factor (ligand) superfamily, member 12	0.446	1.957	6.85E-04	0.071
RUNDC1	RUN domain containing 1	0.445	2.148	5.48E-04	0.069
TTBK2	tau tubulin kinase 2	0.444	1.971	6.64E-04	0.071
SFXN3	sideroflexin 3	0.443	1.589	1.29E-03	0.098
LMO7	LIM domain 7	0.443	2.073	4.83E-04	0.066
IGLL5	immunoglobulin lambda-like polypeptide 5	0.441	3.061	3.72E-04	0.059
TYW1	tRNA-yW synthesizing protein 1 homolog (S, cerevisiae)	0.440	2.195	4.61E-04	0.065
TXNDC5	thioredoxin domain containing 5 (endoplasmic reticulum)	0.438	2.072	6.50E-04	0.071
KIF5C	kinesin family member 5C	0.438	1.973	5.53E-04	0.069
ARL17A	ADP-ribosylation factor-like 17A	0.438	7.587	3.00E-05	0.024
WDR46	WD repeat domain 46	0.433	3.313	1.22E-04	0.049
FER	fer (fps/fes related) tyrosine kinase	0.432	1.709	1.07E-03	0.091
CD40LG	CD40 ligand	0.431	2.076	4.94E-04	0.066
LHX4-AS1	LHX4 antisense RNA 1	0.429	2.390	2.09E-04	0.052
NAAA	N-acylethanolamine acid amidase	0.424	3.089	8.08E-05	0.041
GEMIN5	gem (nuclear organelle) associated protein 5	0.424	1.764	6.21E-04	0.070
RHOF	ras homolog family member F (in filopodia)	0.423	1.482	1.26E-03	0.097
SH3PXD2A	SH3 and PX domains 2A	0.419	2.019	4.66E-04	0.065
MTHFD1	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	0.419	1.612	5.67E-04	0.069
ZBTB22	zinc finger and BTB domain containing 22	0.414	2.056	2.58E-04	0.052
ST3GAL4-AS1	ST3GAL4 antisense RNA 1 (head to head)	0.414	2.015	5.36E-04	0.069
TRMT44	tRNA methyltransferase 44 homolog (S, cerevisiae)	0.414	1.197	1.14E-03	0.093
RING1	ring finger protein 1	0.413	3.144	4.42E-05	0.029
GTF2E1	general transcription factor IIE, polypeptide 1, alpha 56kDa	0.411	1.083	1.14E-03	0.093
RDH13	retinol dehydrogenase 13 (all-trans/9-cis)	0.410	4.235	3.31E-05	0.024
ZNF765	zinc finger protein 765	0.409	1.016	1.20E-03	0.095
TUBGCP5	tubulin, gamma complex associated protein 5	0.406	2.407	8.88E-05	0.043
RHD	Rh blood group, D antigen	0.403	2.908	2.16E-04	0.052
PRR29	proline rich 29	0.403	1.702	4.88E-04	0.066

ARL17B	ADP-ribosylation factor-like 17B	0.402	4.903	7.52E-06	0.009
SERF1B	small EDRK-rich factor 1B (centromeric)	0.400	2.490	1.88E-04	0.052
SERF1A	small EDRK-rich factor 1A (telomeric)	0.400	2.490	1.88E-04	0.052
ZNF772	zinc finger protein 772	0.397	1.076	8.39E-04	0.080
DDX56	DEAD (Asp-Glu-Ala-Asp) box helicase 56	0.397	1.736	2.25E-04	0.052
RFX5	regulatory factor X, 5 (influences HLA class II expression)	0.396	1.944	1.26E-04	0.049
CCDC77	coiled-coil domain containing 77	0.396	1.047	1.17E-03	0.094
TTC27	tetratricopeptide repeat domain 27	0.395	1.591	2.77E-04	0.052
MED22	mediator complex subunit 22	0.395	1.404	7.10E-04	0.072
GLMP	glycosylated lysosomal membrane protein	0.395	0.933	1.37E-03	0.099
PAAF1	proteasomal ATPase-associated factor 1	0.394	0.690	1.37E-03	0.099
RASGRP3	RAS guanyl releasing protein 3 (calcium and DAG-regulated)	0.393	1.974	2.11E-04	0.052
LOC100505549	uncharacterized LOC100505549	0.392	1.567	2.44E-04	0.052
KAT2A	K(lysine) acetyltransferase 2A	0.390	0.695	1.32E-03	0.098
MPI	mannose phosphate isomerase	0.385	1.383	5.94E-04	0.070
SNAPC5	small nuclear RNA activating complex, polypeptide 5, 19kDa	0.385	1.038	6.58E-04	0.071
LOC100133315	transient receptor potential cation channel, subfamily C, member 2-like	0.384	0.825	9.48E-04	0.087
CCHCR1	coiled-coil alpha-helical rod protein 1	0.381	1.425	4.49E-04	0.065
CXXC5	CXXC finger protein 5	0.380	1.305	2.82E-04	0.052
ENTPD6	ectonucleoside triphosphate diphosphohydrolase 6 (putative)	0.380	1.173	5.38E-04	0.069
USP21	ubiquitin specific peptidase 21	0.379	1.280	3.50E-04	0.058
KRBA2	KRAB-A domain containing 2	0.376	0.925	7.42E-04	0.074
MLYCD	malonyl-CoA decarboxylase	0.375	0.567	1.03E-03	0.091
MYO1E	myosin IE	0.375	1.104	7.19E-04	0.072
NR1D1	nuclear receptor subfamily 1, group D, member 1	0.373	1.215	3.12E-04	0.056
NCR1	natural cytotoxicity triggering receptor 1	0.371	1.268	7.15E-04	0.072
RIN2	Ras and Rab interactor 2	0.369	1.058	3.74E-04	0.059
SLC4A10	solute carrier family 4, sodium bicarbonate transporter, member 10	0.368	1.681	1.39E-04	0.049
LILRA4	leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 4	0.368	1.416	6.49E-04	0.071
SNAPIN	SNAP-associated protein	0.365	1.040	6.54E-04	0.071
SERGEF	secretion regulating guanine nucleotide exchange factor	0.365	0.694	7.86E-04	0.077
CD72	CD72 molecule	0.362	0.830	6.04E-04	0.070
ZNF544	zinc finger protein 544	0.362	0.774	6.26E-04	0.070
PRIM1	primase, DNA, polypeptide 1 (49kDa)	0.362	1.246	4.10E-04	0.062
LCMT2	leucine carboxyl methyltransferase 2	0.359	0.167	1.39E-03	0.099
PCDH12	protocadherin 12	0.358	0.651	1.25E-03	0.096
CENPO	centromere protein O	0.358	0.729	6.84E-04	0.071
DEXI	Dexi homolog (mouse)	0.355	0.500	1.20E-03	0.095
LOC101927354	uncharacterized LOC101927354	0.354	-0.514	1.36E-03	0.099
ZNF304	zinc finger protein 304	0.353	0.359	1.13E-03	0.093
TRIM35	tripartite motif containing 35	0.352	1.792	5.03E-05	0.029
IL21R-AS1	IL21R antisense RNA 1	0.351	1.149	2.03E-04	0.052
CD79A	CD79a molecule, immunoglobulin-associated alpha	0.350	1.188	2.80E-04	0.052
IGSF8	immunoglobulin superfamily, member 8	0.349	-0.032	1.28E-03	0.098

ERI2	ERI1 exoribonuclease family member 2	0.349	-0.104	1.04E-03	0.091
DNAJC17	DnaJ (Hsp40) homolog, subfamily C, member 17	0.344	-0.237	1.18E-03	0.095
KCNQ5	potassium channel, voltage gated KQT-like subfamily Q, member 5	0.343	0.334	1.30E-03	0.098
NUDCD1	NudC domain containing 1	0.341	0.457	4.61E-04	0.065
FAM226A	family with sequence similarity 226, member A (non-protein coding)	0.337	1.046	2.13E-04	0.052
FAM226B	family with sequence similarity 226, member B (non-protein coding)	0.337	1.046	2.13E-04	0.052
DDX49	DEAD (Asp-Glu-Ala-Asp) box polypeptide 49	0.330	0.199	5.12E-04	0.067
PPIAL4D	peptidylprolyl isomerase A (cyclophilin A)-like 4D	0.330	0.806	1.69E-04	0.052
PPIAL4F	peptidylprolyl isomerase A (cyclophilin A)-like 4F	0.330	0.806	1.69E-04	0.052
PPIAL4E	peptidylprolyl isomerase A (cyclophilin A)-like 4E	0.330	0.806	1.69E-04	0.052
NDUFB7	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 7, 18kDa	0.330	-0.480	1.39E-03	0.099
ZNF583	zinc finger protein 583	0.330	0.047	6.19E-04	0.070
KLF16	Kruppel-like factor 16	0.328	-0.117	1.32E-03	0.098
ZNF540	zinc finger protein 540	0.328	-0.347	1.01E-03	0.090
CD83	CD83 molecule	0.325	-0.294	1.36E-03	0.099
LOC727751	golgin A2 pseudogene	0.323	-0.112	8.80E-04	0.083
ELAC1	elaC ribonuclease Z 1	0.320	-0.427	1.12E-03	0.093
LOC100507639	uncharacterized LOC100507639	0.319	1.914	2.11E-05	0.021
SFXN2	sideroflexin 2	0.319	-0.043	9.41E-04	0.087
CCDC152	coiled-coil domain containing 152	0.318	-0.181	7.98E-04	0.077
C5orf34	chromosome 5 open reading frame 34	0.317	-0.495	6.85E-04	0.071
KIAA0895L	KIAA0895-like	0.316	1.159	3.37E-05	0.024
LINC01336	long intergenic non-protein coding RNA 1336	0.312	-0.300	5.43E-04	0.069
ZFYVE9	zinc finger, FYVE domain containing 9	0.312	0.063	6.32E-04	0.070
BLOC1S4	biogenesis of lysosomal organelles complex-1, subunit 4, cappuccino	0.310	-0.154	9.20E-04	0.085
PPIAL4C	peptidylprolyl isomerase A (cyclophilin A)-like 4C	0.310	-0.254	3.48E-04	0.058
MPDU1	mannose-P-dolichol utilization defect 1	0.310	0.372	7.86E-04	0.077
DDX51	DEAD (Asp-Glu-Ala-Asp) box polypeptide 51	0.305	0.616	1.01E-04	0.045
RBAK-RBAKDN	RBAK-RBAKDN readthrough	0.305	-0.369	3.36E-04	0.058
DDX12P	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 12, pseudogene	0.301	-0.057	5.05E-04	0.067
TUBA8	tubulin, alpha 8	0.299	-0.670	3.71E-04	0.059
SSSCA1	Sjogren syndrome/scleroderma autoantigen 1	0.298	-0.540	4.39E-04	0.065
UBE2E2	ubiquitin-conjugating enzyme E2E 2	0.297	-0.156	2.85E-04	0.052
FAM223A	family with sequence similarity 223, member A (non-protein coding)	0.295	0.705	2.56E-04	0.052
FAM223B	family with sequence similarity 223, member B (non-protein coding)	0.295	0.705	2.56E-04	0.052
SNHG21	small nucleolar RNA host gene 21	0.293	-0.778	3.41E-04	0.058
MUC20	mucin 20, cell surface associated	0.292	2.497	2.81E-07	0.001
C2	complement component 2	0.290	1.360	2.34E-04	0.052
ZNF256	zinc finger protein 256	0.288	-0.297	4.10E-04	0.062
AMER1	APC membrane recruitment protein 1	0.287	0.778	3.47E-05	0.024
C3orf35	chromosome 3 open reading frame 35	0.287	-0.566	4.03E-04	0.062
LOC102723927	uncharacterized LOC102723927	0.286	1.720	1.58E-06	0.004
SLC25A23	solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 23	0.284	0.342	1.39E-04	0.049

OR2T3	olfactory receptor, family 2, subfamily T, member 3	0.284	-0.812	2.87E-04	0.052
RPS14P3	ribosomal protein S14 pseudogene 3	0.281	-0.270	1.50E-04	0.052
MGC70870	C-terminal binding protein 2 pseudogene	0.281	-0.546	3.63E-04	0.059
OVCH1	ovochymase 1	0.278	-0.523	9.04E-04	0.085
SSSCA1-AS1	SSSCA1 antisense RNA 1 (head to head)	0.275	-0.223	2.32E-04	0.052
SLC35F2	solute carrier family 35, member F2	0.270	-0.895	1.33E-04	0.049
STX8	syntaxin 8	0.262	-0.512	1.99E-04	0.052
PAX8-AS1	PAX8 antisense RNA 1	0.259	0.761	9.30E-06	0.010
NLE1	notchless homolog 1 (Drosophila)	0.256	-0.062	5.76E-05	0.032
ERBB2	erb-b2 receptor tyrosine kinase 2	0.245	-0.464	4.70E-05	0.029
ACKR2	atypical chemokine receptor 2	0.219	0.256	2.36E-04	0.052
YBEY	ybeY metallopeptidase (putative)	0.217	-0.407	9.18E-05	0.043
ALDOC	aldolase C, fructose-bisphosphate	0.216	0.007	2.53E-05	0.023
MIR4646	microRNA 4646	0.205	0.058	4.68E-06	0.006
MTUS1	microtubule associated tumor suppressor 1	0.195	0.298	3.50E-06	0.005
MUC12	mucin 12, cell surface associated	0.194	0.925	3.16E-07	0.001
NDUFA6-AS1	NDUFA6 antisense RNA 1 (head to head)	0.184	1.098	6.65E-05	0.035
ZNF382	zinc finger protein 382	0.177	-0.130	3.19E-06	0.005
MIR8061	microRNA 8061	0.162	0.023	2.07E-06	0.004
BTNL3	butyrophilin-like 3	0.161	1.818	1.88E-07	0.001
C6orf10	chromosome 6 open reading frame 10	0.142	0.508	8.67E-08	0.001

Supplemental Table 3: Target mRNAs of miRNA-340 shown to be significantly negative associated with maternal GDM in the screening group. Fold changes (FC) are shown in the 3rd and logarithmic counts per million (logCPM) are shown in the 4th column of the table. Unadjusted P-values and false discovery rates (FDR) of the paired analysis are indicated in the 5th and 6th column.

Symbol	Name	FC	logCPM	P	FDR
GRB10	growth factor receptor-bound protein 10	0.549	1.748	7.93E-03	0.1486
PAIP1	poly(A) binding protein interacting protein 1	0.509	0.726	4.33E-02	0.1973
CRY2	cryptochrome circadian clock 2	0.559	1.904	1.22E-02	0.1614
ID2	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	0.544	2.518	9.49E-03	0.1547
PITPNB	phosphatidylinositol transfer protein, beta	0.594	3.656	1.09E-02	0.1582
SPRY3	sprouty homolog 3 (Drosophila)	0.644	2.767	2.50E-02	0.1789
SCARB2	scavenger receptor class B, member 2	0.613	3.027	3.92E-02	0.1941

Supplemental Table 4: mRNAs associated with maternal BMI within the screening population. Supplemental Table 4 shows mRNAs significantly (P<0.05, false discovery rate (FDR)<0.1) negative associated with maternal BMI. An unpaired analysis was conducted and adjusted for gestational diabetes, age, maternal weight gain, and pregnancy week. Fold changes (FC) are shown in the 3rd and logarithmic counts per million (logCPM), indicating the relative abundance of the transcript, are shown in the 4th column of the table. P-values and FDR as measures of significance of group differences are indicated in the 5th and 6th column.

Symbol	Name	FC	logCPM	P	FDR
C6orf10	chromosome 6 open reading frame 10	0.760	0.521	1.11E-05	0.021
NCR1	natural cytotoxicity triggering receptor 1	0.823	1.271	1.18E-06	0.003
RMI2	RecQ mediated genome instability 2	0.842	-0.497	1.57E-04	0.083
LOC727751	golgin A2 pseudogene	0.842	-0.069	9.65E-05	0.070
CDKN1C	cyclin-dependent kinase inhibitor 1C (p57. Kip2)	0.848	-0.321	1.16E-04	0.070
UBE2E2	ubiquitin-conjugating enzyme E2E 2	0.849	-0.140	1.16E-04	0.070

RPH3A	rabphilin 3A	0.858	3.037	1.55E-06	0.003
HIST2H4B	histone cluster 2. H4b	0.862	0.282	1.23E-04	0.070
HIST2H4A	histone cluster 2. H4a	0.862	0.282	1.23E-04	0.070
QDPR	quinoid dihydropteridine reductase	0.872	-0.405	2.00E-04	0.097
NCAPG2	non-SMC condensin II complex. subunit G2	0.873	0.445	6.02E-05	0.067
ZNF486	zinc finger protein 486	0.877	2.150	1.01E-04	0.070
S100P	S100 calcium binding protein P	0.877	1.854	6.12E-05	0.067
DEFA1B	defensin. alpha 1B	0.890	9.338	8.70E-05	0.070
DEFA1	defensin. alpha 1	0.890	9.337	8.66E-05	0.070
NOL12	nucleolar protein 12	1.083	2.110	6.96E-05	0.068
PEX14	peroxisomal biogenesis factor 14	1.092	0.735	1.92E-04	0.097
BTBD6	BTB (POZ) domain containing 6	1.093	1.727	2.09E-04	0.098
PGAP3	post-GPI attachment to proteins 3	1.108	0.971	2.21E-05	0.037
ITGB3	integrin. beta 3 (platelet glycoprotein IIIa. antigen CD61)	1.117	5.310	6.51E-05	0.067
ZNF775	zinc finger protein 775	1.135	0.350	5.07E-05	0.068
CCL3L1	chemokine (C-C motif) ligand 3-like 1	1.136	2.183	1.22E-08	0.001
DDX11L10	DEAD/H (Asp-Glu-Ala-Asp/His) box helicase 11 like 10	1.142	1.913	1.27E-04	0.071
CCL3L3	chemokine (C-C motif) ligand 3-like 3	1.150	2.052	1.27E-09	0.001
SLC12A1	solute carrier family 12 (sodium/potassium/chloride transporter). member 1	1.289	3.886	1.83E-07	0.001

Supplemental Table 5: Plasma miRNAs negatively associated with maternal GDM. Supplemental Table 5 shows miRNAs significantly (P<0.05, false discovery rate (FDR)<0.1) negative associated with maternal GDM in the plasma miRNA screening group. A multiple linear regression analysis (confounding covariates: BMI, gestational age, fetal sex, and maternal weight gain) miRNAs were sorted by fold change (FC), shown in the 2nd column of the table. Unadjusted and adjusted P-values are indicated in the 3rd and 4th column.

Name	FC	P	P adjusted
miR-95-3p	0.99	3.339E-03	2.292E+05
miR-3192-3p	0.97	7.258E-04	8.721E+04
miR-660-5p	0.96	2.254E-03	1.767E+05
miR-147b	0.95	8.219E-04	8.972E+04
miR-142-3p	0.95	5.939E-04	7.499E+04
miR-4497	0.94	6.636E-03	3.774E+05
let-7b-5p	0.93	2.854E+01	1.063E+03
miR-375	0.92	3.674E-05	8.508E+03
miR-125b-5p	0.91	3.769E-05	8.508E+03
miR-452-5p	0.91	1.112E-05	3.173E+03
miR-22-3p	0.90	6.563E-03	3.761E+05
miR-215-5p	0.89	5.228E-03	3.166E+05
miR-185-5p	0.87	7.685E-03	4.241E+05
miR-106b-3p	0.87	2.890E-03	2.132E+05
miR-1287-5p	0.86	4.720E-03	2.955E+05
miR-24-2-5p	0.86	5.332E-05	1.019E+04
miR-203a-3p	0.85	3.262E-03	2.292E+05
miR-203b-5p	0.85	3.262E-03	2.292E+05
miR-100-5p	0.85	3.592E-04	5.146E+04
miR-339-3p	0.84	8.917E-03	4.566E+05
miR-1250-5p	0.83	3.415E-03	2.292E+05
miR-182-5p	0.82	2.442E+01	9.576E+01
miR-25-3p	0.81	8.080E-05	1.433E+03

miR-320e	0.81	3.371E-04	5.023E+04
miR-3615	0.81	1.136E-03	1.072E+05
miR-501-3p	0.81	1.593E-03	1.348E+04
miR-4665-5p	0.80	1.567E-03	1.342E+04
miR-576-3p	0.78	1.326E-03	1.162E+05
miR-1228-5p	0.76	3.073E-03	2.222E+05
miR-1307-3p	0.75	1.548E-03	1.341E+05
miR-320d	0.74	9.979E-04	1.006E+05
miR-16-2-3p	0.74	1.056E-03	1.035E+05
miR-6877-5p	0.74	3.530E-03	2.348E+05
miR-194-5p	0.72	5.751E-03	3.428E+05
miR-629-5p	0.72	7.524E-03	4.214E+05
miR-6820-3p	0.72	2.798E-03	2.106E+05
miR-320a	0.71	9.348E+01	3.028E+03
miR-23a-5p	0.70	2.659E-04	4.215E+04
miR-516b-5p	0.70	4.092E-03	2.628E+04
miR-17-3p	0.70	3.367E-05	8.360E+03
miR-193b-5p	0.70	7.587E-04	8.972E+04
miR-2110	0.69	3.766E-04	5.195E+04
miR-526b-5p	0.69	8.239E-03	4.436E+05
miR-629-3p	0.69	8.949E-03	4.566E+05
miR-486-3p	0.69	1.639E+00	1.221E+02
miR-486-5p	0.69	1.639E+00	1.221E+02
miR-512-3p	0.68	1.323E-03	1.162E+05
miR-1306-3p	0.67	3.070E-03	2.222E+05
miR-320c	0.67	9.817E-05	1.701E+04
miR-4504	0.66	8.794E-03	4.550E+05
miR-362-5p	0.66	6.884E-03	3.885E+04
miR-520c-5p	0.65	3.711E-03	2.425E+05
miR-320b	0.65	1.053E-05	3.137E+03
miR-518d-5p	0.64	3.406E-03	2.292E+05
miR-526a	0.64	3.406E-03	2.292E+05
miR-210-3p	0.64	8.307E-04	8.972E+04
miR-378a-3p	0.62	2.659E-03	2.036E+05
miR-3200-5p	0.62	8.335E-03	4.436E+05
miR-671-5p	0.61	7.230E-05	1.347E+04
miR-4429	0.61	4.296E-04	5.819E+04
miR-516a-5p	0.61	1.271E-03	1.162E+05
miR-193a-5p	0.60	9.939E-01	5.696E+02
miR-4516	0.57	9.342E-04	9.667E+04
miR-4320	0.56	8.710E-03	4.539E+05
miR-519a-5p	0.56	1.919E-01	3.233E+01
miR-522-5p	0.56	2.604E-01	3.233E+01
miR-518e-5p	0.56	2.349E-01	3.233E+01
miR-519b-5p	0.56	2.349E-01	3.233E+01
miR-519c-5p	0.56	2.349E-01	3.233E+01

miR-523-5p	0.56	2.349E-01	3.233E+01
miR-183-5p	0.53	3.977E+01	1.411E+03
miR-520a-3p	0.53	2.066E+01	8.552E+02
miR-4508	0.52	7.848E-04	8.972E+04
miR-451b	0.51	6.188E-04	7.659E+04
miR-33b-5p	0.51	9.974E-03	4.794E+05
miR-378c	0.51	2.916E-04	4.526E+04
miR-144-5p	0.50	5.453E-04	7.127E+04
miR-483-5p	0.49	3.177E-04	4.831E+04
miR-378d	0.48	2.678E-03	2.036E+05
miR-4732-5p	0.46	1.249E-04	2.067E+04
miR-1180-3p	0.46	5.560E-04	7.142E+04
miR-4532	0.45	1.975E-03	1.653E+05
miR-4732-3p	0.39	3.490E-01	3.715E+01
miR-5001-3p	0.32	7.776E-03	4.260E+04

Supplemental Table 6: Plasma miRNAs positively associated with maternal GDM. Supplemental Table 6 shows miRNAs significantly (P<0.05, false discovery rate (FDR)<0.1) positive associated with maternal GDM in the plasma miRNA screening group. A multiple linear regression analysis (confounding covariates: BMI, gestational age, fetal sex, and maternal weight gain) was conducted. miRNAs are sorted by fold change (FC), shown in the 2nd column of the table. Unadjusted and adjusted P-values are indicated in the 3rd and 4th column.

Name	FC	P	P adjusted
miR-503-5p	1.009	6.36E-03	3.70E-02
miR-7704	1.028	2.71E-05	6.96E-04
miR-6729-3p	1.032	2.23E-04	3.61E-03
miR-191-5p	1.036	1.11E-03	1.06E-02
miR-2355-3p	1.042	8.64E+01	2.93E-04
miR-4683	1.055	8.33E-03	4.44E-02
miR-129-5p	1.074	8.71E-03	4.54E-02
miR-425-3p	1.080	4.95E-03	3.07E-02
miR-6087	1.110	1.01E-03	1.01E-02
miR-28-5p	1.121	3.96E-05	8.67E-04
miR-296-3p	1.142	9.26E-04	9.67E-03
miR-3198	1.146	9.72E-03	4.70E-02
miR-3198	1.146	9.72E-03	4.70E-02
miR-206	1.151	3.76E-04	5.20E-03
miR-652-3p	1.193	3.79E-03	2.45E-02
miR-92a-3p	1.223	1.47E+01	7.32E-05
miR-3177-3p	1.234	9.31E-03	4.63E-02
miR-30d-5p	1.251	6.27E-04	7.66E-03
miR-221-3p	1.262	1.29E-03	1.16E-02
miR-92b-3p	1.268	1.68E+01	7.84E-05
miR-24-3p	1.287	9.20E-03	4.60E-02
miR-3074-5p	1.287	9.16E-03	4.60E-02
miR-150-5p	1.290	4.94E-04	6.57E-03
miR-10a-5p	1.313	1.11E+01	5.91E-05

miR-199a-5p	1.321	9.69E-03	4.70E-02
let-7d-3p	1.343	8.17E-03	4.44E-02
miR-30b-5p	1.345	4.29E-03	2.71E-02
miR-128-3p	1.358	2.88E-03	2.13E-02
miR-584-5p	1.386	3.59E-04	5.15E-03
miR-625-3p	1.394	2.16E-03	1.73E-02
miR-625-5p	1.394	2.13E-03	1.72E-02
miR-128-3p	1.433	2.21E-03	1.75E-02
miR-654-3p	1.463	3.68E-03	2.43E-02
let-7c-5p	1.493	1.01E-05	3.13E-04
miR-10b-5p	1.520	6.13E-01	5.71E+01
miR-27b-3p	1.534	1.00E-03	1.01E-02
miR-30e-3p	1.587	2.46E-05	6.53E-04
miR-15b-3p	1.641	2.10E-03	1.72E-02
miR-493-5p	1.690	5.06E-03	3.09E-02
miR-766-3p	1.809	4.30E-05	8.90E-04
miR-23b-3p	1.826	8.35E-04	8.97E-03
miR-23a-3p	1.868	3.73E-05	8.51E-04
miR-361-5p	1.869	5.75E-03	3.43E-02
miR-199a-3p	1.870	8.42E-04	8.97E-03
miR-199b-3p	1.871	8.43E-04	8.97E-03
miR-224-5p	1.892	6.48E-03	3.74E-02
let-7f-5p	2.090	5.81E-03	3.43E-02
miR-223-3p	2.104	4.93E-05	9.88E-04
miR-331-3p	2.157	2.11E-03	1.72E-02
miR-628-3p	2.313	8.49E-03	4.49E-02
miR-574-3p	2.345	2.45E-03	1.90E-02
miR-4523	2.581	7.61E-05	1.38E-03

Supplemental Table 7: Primer sequences of qPCR experiments

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
APL	ATAAGGGACCCATGCCTTTC	CCTCCAGAGAAGCAGACCAA
APJ	CATCTTCGTCAACATGTACGC	GGTAGCGGTCGAAGCTGA
CRY2	CAAGTCCTTCAGTGGGGA AC	CAAGTCCTTCAGTGGGGAAC
FABP3	AGCAGATGACAGGAAGGTCAA	CAATTAGCTCCCGCACAAGT
FABP4	CCTTTAAAAATACTGAGATTTCCTTCA	GGACACCCCCATCTAAGGTT
FAT	TGCCTATTCTTTGGCTTAATGAG	TTACTTGACTTCTGAACATGTTTGC
GRB10	TTCTGGTAAAGGAGC ATTCCA	AGGACGAGCAAACCAGGAC
ID2	CTGGACTCGC ATCCCACTAT	TAACTCAGAAGGGAATTCAGAAGC
IL1B	CTGTCCTGCGTGTTGAAAGA	TTGGGTAATTTTTGGGATCTACA
IL6	CAGGAGCCCAGCTATGAACT	GAAGGCAGCAGGCAACAC
IL8	AGACAGCAGAGCACACAAGC	AGGAAGGCTGCCAAGAGAG
LIPG	ATTGCCAGGATGCTCGACT	GTGGACATTCCCGAGAGAAA
LPL	AGGAGCATTACCCAGTGTCC	CCAAGGCTGTATCCCAAGAG
PAIP1	AATCC TCACAACCATCCTCATAG	GAACTCGGAGTCAGCAATGG

PITPNB	CGAGACTCAGAAAGAACTAGAAACA	TGACCCTACAGGGGACTCAT
PLIN2	CGTTGCAGTTGATCCACAA	GGATACTGGTCCTTTGTACTGAGAT
RPS13	CCCCACTTGGTTGAAGTTGA	ACACCATGTGAATCTCTCAGGA
RPS13	CCCCACTTGGTTGAAGTTGA	ACACCATGTGAATCTCTCAGGA
SCARB	GGCCGATGCTGCTTCTAC	CAAATGCCTCAGTACCATTCC
SLC27A1	GTCGTCCTCCGCAAGAAAT	TCCCCGATGTACTGAACCAC
SLC27A4	TGCCTGAGCTGCACAAAA	AGAACAGCGGGTCTTTCACA
SPRY3	AAATCATCTGTTAGCCCCTAACTC-	TCATATGAAATGTATCAA GGAACCA
TLR2	CTCTCGGTGTCGGAATGTC	AGGATCAGCAGGAACAGAGC
TLR4	AGCCATGGCCTTCCTCTC	TTCAGCTCCATGCATTGATAA
TNF-α	CAGCCTCTTCTCCTTCCTGAT	GCCAGAGGGCTGATTAGAGA
miRNA gene		**Primer sequence**
RNU6B		Not published
let-7g-5p		UGAGGUAGUAGUUUGUACAGUU
miRNA-142-5p		CAUAAAGUAGAAAGCACUACU
miRNA-143-3p		UGAGAUGAAGCACUGUAGCUC
miRNA-19a-3p		UGUGCAAAUCUAUGCAAAACUGA
miRNA-19b-3p		UGUGCAAAUCCAUGCAAAACUGA
miRNA-340-5p		UCCGUCUCAGUUACUUUAUAGC