# BMI-Independent Effects of Gestational Diabetes on Human Placenta

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**Purpose:** Recently, alterations in maternal lipid metabolism were associated with gestational diabetes mellitus (GDM). However, detailed plasma lipid profiles and their relevance for placental and fetal metabolism are currently not understood.

**Methods:** Maternal and placental lipid profiles were characterized in women with GDM and women with normal glucose tolerance (NGT). Inflammatory gene expression was compared in placentas and primary term trophoblasts between the groups. In addition, trophoblasts were stimulated with nonesterified fatty acids (NEFAs), and effects on gene expression were quantified. Finally, placental macrophage content and cord blood concentrations of inflammatory parameters and NEFAs were compared between women with GDM and women with NGT with similar body mass index (BMI).

**Results:** Palmitate and stearate levels were elevated in both maternal plasma and placental tissue of women with GDM. Placental GDM-associated elevations of IL6, IL8, and TLR2 expression were reflected in trophoblasts derived from women with GDM. Stimulation of primary trophoblasts with palmitate led to increased mRNA expression and protein release of the cytokine IL6 and the chemokine IL8. In line with this, elevated amounts of CD68-positive cells were quantified in the placental tissue of women with GDM. No GDM-associated elevations in a range of inflammatory parameters and NEFAs in cord blood of NGT vs GDM neonates was found.

**Conclusions:** GDM, independently of BMI, altered maternal plasma NEFAs and the placental lipid profile. GDM was associated with trophoblast and whole-placenta lipoinflammation; however, this was not accompanied by elevated concentrations of inflammatory cytokines or NEFAs in neonatal cord blood. (***J Clin Endocrinol Metab*** **103: 3299–3309, 2018)**

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Abbreviations: BMI, body mass index; CE, cholesteryl ester; DG, diacylglycerol; FA, fatty acid; FC, fold change; GDM, gestational diabetes mellitus; IFN, interferon; MIP, macrophage inflammatory protein; NEFA, nonesterified fatty acid; NGT, normal glucose tolerance; OGTT, oral glucose tolerance test; PEACHES, Programming of Enhanced Adiposity Risk in Childhood – Early Screening; PL, phospholipid; SFA, saturated nonesterified fatty acid; T2D, type 2 diabetes; TG, triacylglycerol; VEGF, vascular endothelial growth factor.

Gestational diabetes mellitus (GDM) influences the future health of the child. It was shown that an increased risk for the development of overweight and diabetes in the adult has its origin in fetal exposure to harmful environments, such as maternal obesity (1) and GDM (2). The subtle consequences of fetal programming may, in the long run, shape an adult phenotype prone to develop obesity and ultimately type 2 diabetes (T2D) (3, 4). Elevated maternal glucose levels in women with type 1 diabetes were shown to increase fetal insulin levels (5). In addition, other factors such as maternal lipids are proposed to modify fetal metabolism (6, 7). *In utero*, these parameters could mediate the offspring's risk for the development of T2D later in life (1, 8). In adult humans, increased levels of nonesterified fatty acids (NEFAs) arising from insulin-resistant hypertrophic adipose tissue have been associated with subclinical inflammation and the development of peripheral and central insulin resistance (9, 10). GDM-associated effects of elevated total placental lipid accumulation (11) could favor a placental state of subclinical inflammation comparable to that seen in adipose tissue and vessel walls of patients with hyperlipidemic T2D (described as lipoinflammation) (12). Indeed, earlier studies showed that GDM- and/or body mass index (BMI)–associated elevated expression of genes was involved in placental fatty acid (FA) uptake and transport (13, 14). Furthermore, genome-wide mRNA-expression studies proposed activation of inflammatory pathways and endothelial reorganization (15, 16). However, none of the studies investigated the influence of GDM independently of maternal BMI. An add-on effect of GDM on adverse fetal outcome has been seen in epidemiological studies (17); however, the molecular mechanisms of GDM, independent of maternal BMI, are not clear.

Therefore, the aim of this work was to investigate the effects of GDM, independent of BMI, on maternal plasma and placental lipid profiles as well as on whole-placenta and trophoblast-specific lipoinflammation, which may be of direct relevance to the fetus. We showed earlier that even subtle changes in FA profiles of different insulin target tissues are associated with the induction of insulin resistance (18). To approximate how lipid-associated alterations of the placenta could contribute to alterations in the *in utero* development of insulin resistance (19, 20), we compared a wide range of inflammatory parameters and NEFAs in cord blood samples of normal glucose tolerant (NGT) and GDM offspring.

## Material and Methods

### Study design and diagnosis of GDM

Pregnant women with NGT and GDM from the ongoing Tübingen Pregnancy study were selected (Table 1). From this cohort, maternal blood was collected during pregnancy (gestational week 27 ± 2) (NGT, N = 18; GDM, N = 18). From another subgroup of the Tübingen Pregnancy study, placental tissue (NGT, N = 19; GDM, N = 11) and fetal cord blood (NGT, N = 11; GDM, N = 7) were collected for cord blood FA profiles [Fig. 1(a)]. For primary cell culture experiments, trophoblasts from the independent Graz trophoblast cohort (NGT, N = 7; GDM, N = 7) were used [Fig. 1(b)]. For quantification of inflammatory parameters, cord blood samples from the independent Programming of Enhanced Adiposity Risk in CHildhood – Early Screening (PEACHES) cohort (NGT, N = 52; GDM, N = 52) were available [Fig. 1(c)] (21).

Participants of the Tübingen Pregnancy and Graz Trophoblast cohorts underwent a five-point 75-g oral glucose tolerance test (OGTT) after overnight fasting. Venous blood samples were obtained at 0, 30, 60, 90, and 120 minutes for determination of plasma glucose level using a bedside glucose analyzer (YSI, Yellow Springs, OH). GDM was diagnosed according to the International Association of the Diabetes and Pregnancy Study Groups recommendations for the diagnosis and classification of hyperglycemia in pregnancy (0-hour glucose level ≥5.11 mmol/L or 1-hour glucose level ≥10.0 mmol/L, or 2-hour glucose level ≥8.5 mmol/L) (22). The PEACHES participants had either a 75-g OGTT or a 50-g glucose challenge test followed by a 75-g OGTT in case of a positive result [1-hour glucose ≥7.8 mmol/L (23)] (21). One woman with GDM from the Tübingen Pregnancy cohort, no women from the Graz Trophoblast cohort, and 16 women from the PEACHES cohort underwent insulin treatment.

Similar maternal BMI of women with NGT and GDM was required for inclusion in the analyses, and previous T1D, T2D, preeclampsia, dyslipidemia, drug abuse; age below 18 years; and psychological disorders were exclusion criteria. All three independent studies were 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. Study work flow and exclusion criteria are shown in Fig. 1.

### Sampling of maternal blood, placental tissue, and cord blood as well as clinical characteristics of study participants

At delivery in women from the Tübingen Pregnancy study, placental tissue (NGT, N = 19; GDM, N = 11) was collected strictly following a high-quality random sampling protocol (24, 25) and was extensively washed (4° to 10°C, 140 mmol/L of NaCl solution, for 30 seconds) immediately. From each placenta, five samples (~1 $cm^3$) were randomly collected. Thereafter, tissue of the surface facing the fetal circulation was separated, cut in smaller pieces (10 to 20 mg), and pooled. Tissue was stored at −80°C until further analysis of RNA, proteins, and FA. Whole cord blood collection was conducted at birth, and serum and plasma were aliquoted and stored at −80°C. Metabolic (OGTT, glucose challenge test) and anthropometric (weight, height) characterization of the women was conducted, and data are shown in Table 1. In addition, information about mode of delivery (spontaneous delivery including vacuum extraction and induction of labor, cesarean section) was collected, as shown in Table 1.

### Determination of the FA profile of different lipid classes in maternal blood, placental tissue, and cord blood

Maternal (NGT, N = 18; GDM, N = 18) and cord blood (NGT, N = 11; GDM, N = 7) plasma as well as placental samples (NGT, N = 8; GDM, N = 8) were collected and processed for determination of FA profiles by strictly following

**Table 1. Anthropometric and Metabolic Characteristics of the Participants**

| | Tübingen Pregnancy Cohort | | | Graz Trophoblast Cohort | | | PEACHES Cohort | | |
|---|---|---|---|---|---|---|---|---|---|
| | NGT | GDM | *P* | NGT | GDM | *P* | NGT | GDM | *P* |
| N | 37 | 29 | — | 7 | 7 | — | 52 | 52 | — |
| Maternal age, y | 31 ± 5 | 34 ± 4 | 0.3 | 33 ± 5 | 30 ± 4 | 0.2 | 33 ± 4 | 33 ± 4 | 0.6 |
| Prepregnancy body mass index, kg/m$^2$ | 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 |
| Midpregnancy BMI, kg/m$^2$ | 27.0 ± 6.0 | 30.6 ± 5.6 | 0.4 | — | — | — | 24.9 ± 2.6 | 25.4 ± 2.9 (N = 49) | 0.3 |
| Gestational age at delivery, wk | 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 level, mmol/L | 4.4 ± 0.3 | 4.8 ± 0.5 | **$3 \times 10^{-5}$** | 4.3 ± 0.3 | 5.4 ± 0.4 | **0.008** | 4.4 ± 0.5 | 5.0 ± 0.5 | **$3 \times 10^{-5}$** |
| 1-h glucose, mmol/L (75-g OGTT) | 7.3 ± 1.3 | 10.5 ± 1.3 | **$6 \times 10^{-14}$** | 5.2 ± 0.8 | 9.0 ± 1.9 | **0.002** | 6.9 ± 1.5 | 9.4 ± 1.8 | **$3 \times 10^{-7}$** |
| 2-h glucose, mmol/L (75-g OGTT) | 6.1 ± 1.1 | 8.4 ± 1.0 | **$6 \times 10^{-13}$** | 5.0 ± 0.6 | 7.3 ± 1.18 | **0.009** | 5.8 ± 1.3 | 7.4 ± 1.5 | **$3 \times 10^{-4}$** |
| 1-h glucose, mmol/L (50-g GCT) | | | | | | | 5.9 ± 1.4 (N = 28) | 8.3 ± 0.6 (N = 2) | **0.02** |
| Women with GDM receiving insulin treatment | 0 (0%)*a* | 1 (9.1%)[a] | — | 0 (0%) | 0 (0%) | — | 0 (0%) | 16 (30.8%) | — |
| Smokers before pregnancy, No. | 2 (10.5)%)[a] | 4 (36.4%)[a] | 0.1 | — | — | — | 6 (11.5%) | 13 (25.5%) | 0.1 |
| Spontaneous delivery, No. | 12 (75.0%)[a,b] | 5 (45.5%)[a] | 0.1 | 5 (71.41%) | 4 (57.1%) | 0.6 | 32 (61.5%)[b] | 33 (63.5%)[b] | 0.8 |
| Cesarean section, No. | 4 (25.0%) (N = 16) | 6 (54.5%) | | 2 (28.6%) | 3 (42.9%) | | 20 (38.5%) | 19 (36.5%) | |
| Female neonates, No. | 6 (17.7%)[a] | 5 (17.2%)[a] | 0.1 | 3 (42.9%) | 0 (0%) | 0.2 | 26 (50%) | 27 (51.9%) | 0.8 |
| Birth weight, g | 3391.8 ± 638.1[a] | 3497.14 ± 286.3[a] | 0.3 | 3068.0 ± 354.0 | 3327.0 ± 322.0 | 0.3 | 3393.5 ± 428.9 | 3309.0 ± 390.0 | 0.3 |
| Cord blood insulin, pmol/L | — | — | — | — | — | — | 15.3 ± 16.2 (N = 51) | 16.5 ± 14.8 | 0.7 |

Anthropometric and metabolic characteristics of the Tübingen Pregnancy cohort, Graz Trophoblast cohort, and PEACHES cohort. From smaller subgroups of the Tübingen 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); all other women (N = 74) had either a 75-g OGTT or a 50-g GCT followed by a 75-g OGTT. Among these, three women in the NGT group had a positive GCT followed by a negative 75-g OGTT. Midpregnancy was defined as 27 ± 2 gestational weeks (Pregnancy cohort) or 22 ± 1 gestational weeks (PEACHES cohort). All data are given as absolute numbers or mean ± SD. Group differences in numeric characteristics were calculated by two-tailed Student *t* test. Group differences in fetal sex, smoking behavior, and mode of delivery (spontaneous delivery or cesarean section) were calculated using a $\chi^2$ test. *P* values ≤0.05 were considered statistically significant and are shown in boldface. Available sample sizes for variables are presented in parentheses when relevant. Variables not collected for a specific cohort are indicated with a dash.

[a]Placenta subgroup (NGT, N = 19; GDM, N = 11).

[b]Includes vacuum extraction (one women with NGT in the Tübingen Pregnancy cohort, three women with NGT and four women with GDM in the PEACHES cohort).

[c]Induction of labor was performed in N = 13 (25%) women in the NGT and GDM group.

preanalytical recommendations described earlier (26). 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 (CEs), diacylglycerols (DGs), NEFAs, phospholipids (PLs), and triacylglycerols (TGs)] using thin-layer chromatography. Methodological details of thin-layer chromatography and further determination of FA profiles by gas chromatography were described earlier (27). 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-n3, C22:6-n3, C24:0, C24:1-n9).

## Primary placental trophoblast experiments

Primary trophoblasts were isolated from the placentas of women with similar maternal BMI (NGT, N = 7; GDM, N = 7). Isolation and quality control human choriogonadotropin β-subunit release, cytokeratin 7, vimentin, and histocompatibility antigen, class I, G-staining was conducted as described previously (28, 29). We observed no reduction in human choriogonadotropin β-subunit levels and only positive staining of cytokeratin 7 (Supplemental Fig. 1). After isolation, cells were kept in DMEM medium (Lonza, Basel, Switzerland) for 48 hours followed by a 24-hour stimulation with medium, 100 μM of palmitate or 100 μM of oleate (Sigma-Aldrich) coupled with 10% BSA solution (Sigma-Aldrich) and corresponding control conditions (30). The BSA concentration (10%) was chosen to provide a surplus of binding sites for the FAs applied. After a stimulation period of 24 hours, cell culture supernatants were collected.

## RNA isolation, cDNA synthesis, and quantitative PCR

RNA was isolated from homogenized placental tissue (N = 30) and trophoblasts (N = 14) using miRNeasy (Qiagen). Reverse transcription and quantitative PCR were described earlier (31). Expression of genes involved in lipid metabolism (FA transporters: *FAT*, *SLC27A1*, *SLC27A4*; FA–binding proteins: *FABP3*, *FABP4*; lipases: *LIPG*, *LPL*; marker for lipid droplets: *PLIN2*) and inflammation *(IL1B*, *IL6*, *IL8*, *TNF*, *TLR2*, *TLR4*) as well as the expression of the stably expressed reference gene *RPS13* was determined using the Light Cycler 450 (Roche, Mannheim,

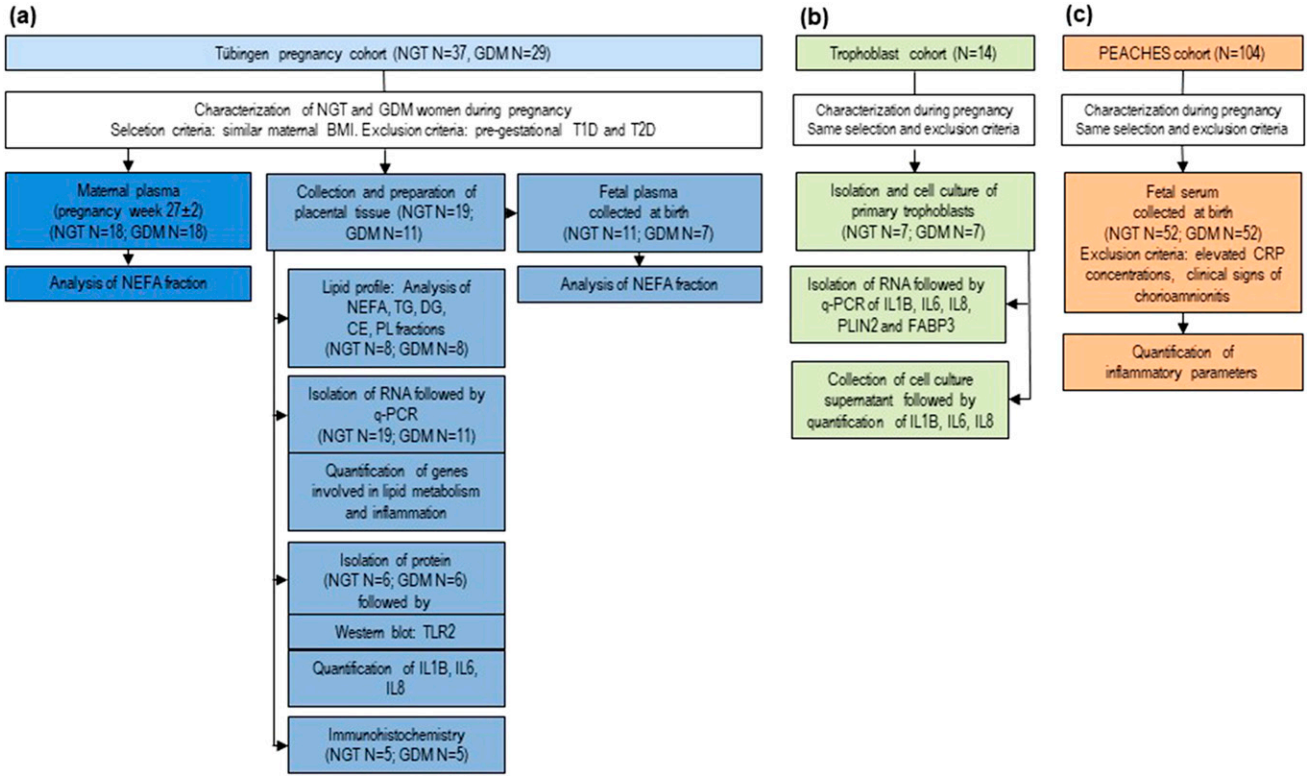


**Figure 1.** Study work flow. Available samples and work flow of (a) the Tübingen Pregnancy cohort, (b) the Graz Trophoblast cohort, and (c) the PEACHES cohort are presented. CE, cholesteryl ester; CRP, C-reactive protein; DG, diacylglycerol; PL, phospholipid; qPCR, quantitative PCR; TG, triacylglycerol.

Germany). Primer sequences and fluorescent probes are shown in Supplemental Table 1.

## Cytokine and chemokine 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) (NGT, N = 6; GDM, N = 6). Tissue pieces were homogenized by Qiashredder (Qiagen) and ultrasonography in protein lysis buffer (10 mM Tris containing complete and PhosSTOP). Total protein concentrations were determined with the Pierce BCA Protein Assay Kit (Thermo Fisher, Waltham, MA). Placental IL1B, IL6, and IL8 protein levels were quantified using Quantikine HS ELISA (R&D, Minneapolis, MN) in duplicates and normalized for total cellular protein. Cytokine and chemokine concentrations in primary trophoblast cell culture supernatants were similarly quantified using Quantikine HS ELISA (R&D) (NGT, N = 7; GDM, N = 7). Inflammatory cytokines in fetal cord blood sera (NGT, N = 52; GDM, N = 52) were quantified using Bio-Plex Pro Human Cytokine-27-plex Kit (Bio-Rad Laboratories, Hercules, CA) following the manufacturers' recommendations. Concentrations outside the standard curve were not considered for statistical analysis, leading to reduced sample sizes: granulocyte-colony stimulating factor (N = 56), GM-CSF (N = 53), IL6 (N = 57), and IL15 (N = 65), interferon (IFN)-g (N = 71), IL8 (N = 98), macrophage inflammatory protein (MIP)-1a (N = 96), regulated on activation (N = 58), and vascular endothelial growth factor (VEGF) (N = 67).

## Protein visualization and quantification by Western blot

Placental tissue was shredded and homogenized in a protein lysis buffer [50 mM HEPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 0.1 mM egtazic acid (Roth, Karlsruhe, Germany), 10% glycerol (Roth), 1% Triton X-100 (Sigma-Aldrich)]. The proteins from tissue lysates were resolved on a 10% Tris-HCl gel and blotted onto nitrocellulose membranes. Unspecific binding sites were blocked with 2% milk TBS-Tween (Sigma-Aldrich) before overnight incubation with primary TLR2 antibody (1:500; Abcam, Cambridge, England). $\beta$-actin (1:1000; Cell Signaling, Danvers, MA) was used as loading control. Incubation with primary antibody was followed by 1-hour incubation with an horseradish peroxidase-coupled secondary antibody (1:3000). Antibodies were diluted in 2% milk in TBS-Tween. Protein bands were detected and quantified with Chemidoc Touch Image laboratory software (Bio-Rad Laboratories).

## Quantification of macrophages

Paraffin-embedded, serial sections of placental tissue (NGT, N = 5; GDM, N = 5) were incubated with a primary antibody against CD68 (1:3000, clone KP-1; Dako, Jena, Germany). The primary antibody was detected using the Opti-View System (Roche-Ventana, Multimer Technology, Basel, Switzerland). Hematoxylin and 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 to those from morphologically comparable sections of placental tissue slides were selected. From each placenta, four pictures from three slides were used for quantification. The percentage of CD68-positive

placental villi related to the total amount of selected villi was calculated.

### Statistical analysis

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, and fetal sex as confounders, and GDM vs NGT as independent variables. The descriptive clinical data presented in Table 1 were analyzed by two-tailed Student *t* test. The data from *in vitro* experiments were analyzed by one-tailed Student *t* test (two-group comparisons) or ANOVA with the Dunnett *post hoc* test (three- and more-group comparisons). Differences with $P \leq 0.05$ were considered statistically significant.

## Results

### Metabolic and anthropometric characterization of pregnant women

The clinical characteristics of the cohorts are shown in Table 1. An OGTT conducted in pregnancy week 27 ± 2 showed significantly increased fasting 1-hour and 2-hour glucose levels in women with GDM compared with the NGT group of the Tübingen Pregnancy cohort ($P \leq 3 \times 10^{-5}$, all). No statistical group differences were found in maternal smoking behavior, mode of delivery ($P = 0.1$ for both), and fetal sex ($P = 0.2$). Clinical characteristics of the Trophoblast cohort and maternal and perinatal characteristics of the analyzed subgroup of the PEACHES cohort were similar (Table 1). Differences between the cohorts in BMI during pregnancy may result in part from different time points of clinical characterization.

### Maternal plasma lipid analysis

In maternal plasma, FA concentrations of the NEFA fraction were analyzed. Total and saturated nonesterified fatty acid (SFA) concentrations were elevated in women with GDM compared with women with NGT ($P \leq 0.05$) [Fig. 2(a); Supplemental Fig. 2(a)]. The most abundant plasma NEFAs were palmitate (C16:0), stearate (C18:0), oleate (C18:1-n9), and linoleate (C18:2-n6) [Fig. 2(b)]. Concentrations of palmitoleate (16:1-n7), stearate, and docosahexaenoate (C22:6-n3) were higher in the plasma of women with GDM than of women with NGT ($P \leq 0.05$) [Fig. 2(b) and 2(c)]. Statistical trends for elevated amounts of palmitate, vaccenate (C18:1-n7), oleate, arachidonate (C20:4-n6), and nervonate (C24:1-n9) were observed ($P \leq 0.1$, all).

### Placental lipid analysis

Analysis of FA concentrations of five lipid classes (CE, DG, PL, NEFA, and TG) from placental homogenates showed that women with GDM had a 1.5-fold increased level of TGs in placental tissue compared with women with NGT and similar BMI ($P = 0.04$) [Fig. 2(d)]. Within the NEFA fraction, main SFA C16:0 and C18:0 were higher by 1.2-fold and 1.4-fold, respectively, in the placental tissue of women with GDM compared with women with NGT ($P = 0.02$ and $P = 0.01$) [Fig. 2(e); Supplemental Fig. 2(b)]. Eicosatrienoate (C20:3-n6) and docosahexaenoate (C22:6-n3) showed statistical trends for reduced levels in GDM placental tissue compared with NGT placental tissue ($P \leq 0.1$) [Fig. 2(f)]. Similarly, reduced amounts of C20:3-n6 were found in the PL fraction in GDM placental tissue ($P = 0.04$). Furthermore GDM-associated elevations of eicosanoate (C20:0; PL fraction) as well as of docosanoate (C22:0; DG fraction) ($P \leq 0.04$) were seen. Differences in CE and TG fractions were absent ($P > 0.05$). Concentrations of each FA in all fractions are shown in Supplemental Table 2.

### Gene expression of whole-placenta tissue

Placental target genes involved in lipid metabolism (*FABP3, FABP4, FAT, LIPG, PLIN2, SLC27A1, SLC27A4*) and inflammation (*IL1B, IL6, IL8, TNF, TLR2, TLR4*) were selected. Increased mRNA expression of *FABP3* [fold change (FC) = 1.4; $P = 0.01$], *IL1B* (FC = 1.6; $P = 0.03$), *IL6* (FC = 2.0; $P = 0.05$), and *TLR2* (FC = 1.1; $P = 0.02$) was detected in placental tissue of women with GDM. Except for a statistical trend for elevated *IL8* ($P = 0.07$) mRNA expression in the tissue of women with GDM, no differences were identified for the other genes investigated ($P > 0.1$). Because the chemokine *IL8* plays a major role in mediation of immune cell infiltration (32), this gene was included in further experiments. Next, we analyzed the association of maternal plasma total NEFA values with placental mRNA expression. Significant positive correlations of maternal total plasma NEFA concentrations and placental *IL1B* ($P = 0.02$), *IL6* ($P = 0.02$), and *TLR2* ($P = 0.05$) mRNA expression were found [Fig. 3(a), 3(b), and 3(d)]. For *IL8*, the association with maternal plasma NEFA concentrations was of borderline significance ($P = 0.08$) [Fig. 3(c)]. Furthermore, significantly elevated protein levels of IL1B and IL6 ($P = 0.03$ for both) [Fig. 3(e) and 3(f)] and statistical trends for elevated IL8 and TLR2 levels ($P \leq 0.08$) were detected in the placental tissue of women with GDM [Fig. 3(g) and 3(h); Supplemental Fig. 3)].

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

Primary human trophoblasts derived from women with GDM and BMI-matched women with NGT were cultured in medium for 48 hours. On the basis of findings in the whole placenta, mRNA expression of *FABP3, IL1B, IL6,* and *TLR2* was quantified. *PLIN2* was included in the analysis (11). *IL1B* mRNA expression signals were at the detection limit (all $C_q$ values ≥35); hence, they were excluded from

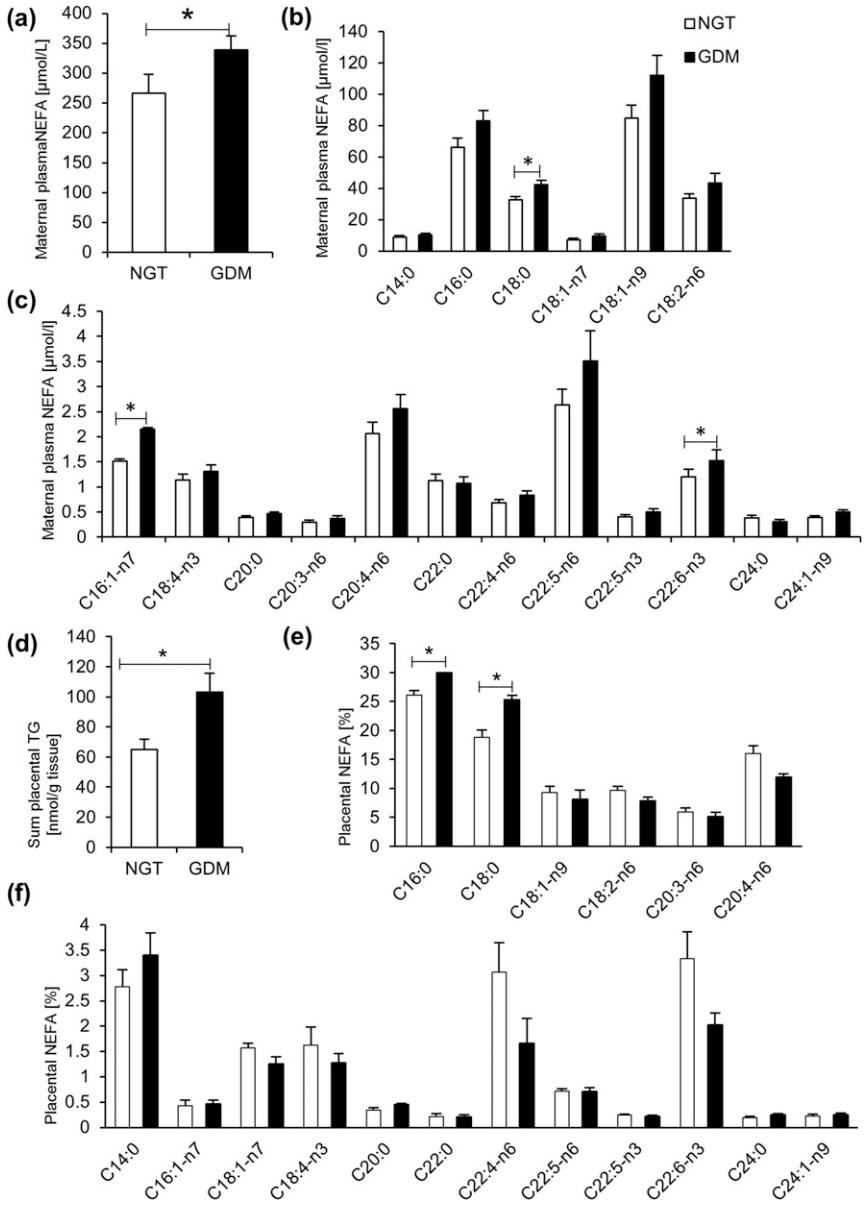


**Figure 2.** Lipid pattern. (a) Sum of maternal plasma NEFAs of women with NGT (N = 18) (white columns) and women with GDM (N = 18) (black columns) and similar BMI. (b) Highly abundant (>5 μmol/L) and (c) less abundant (≤5 μmol/L) NEFA species of maternal plasma of women with NGT and women with GDM. (d) Sum of placental triacylglycerols (TGs) of women with NGT (N = 8) and women with GDM (N = 8) and similar BMI. (e) Highly abundant (>5%) and (f) less abundant (≤5%) NEFA species of placental tissues of women with NGT and women with GDM. Shown are means ± SEM. Multiple linear regression analyses were conducted. Significant changes ($P \leq 0.05$) are marked with an asterisk.

further analysis. Elevated mRNA expression for *IL6* (N = 12; $P = 0.02$), *TLR2* (N = 10; $P = 0.05$), and *PLIN2* (N = 12; $P = 0.03$) [Fig. 4(a), 4(c), and 4(d)] and a statistical trend for elevated expression of *IL8* (N = 12; $P = 0.07$) [Fig. 4(b)] were found in trophoblasts derived from women with GDM (reduced group sizes due to the exclusion of individual data with $C_q$ values ≥35). *FABP3* mRNA (N = 8; $P = 0.2$) did not differ between the groups [Fig. 4(e)].

On the basis of findings of increased amounts of SFAs and altered mRNA expression in the whole-placenta tissue of women with GDM, we investigated possible lipoinflammatory effects of defined NEFAs on trophoblast mRNA expression. To this end, primary human trophoblasts derived from women with GDM and BMI-matched NGT controls were stimulated with the SFA palmitate (C16:0) and the monounsaturated NEFA oleate (C18:1-n9), respectively, and a control condition. Palmitate was shown earlier to be highly lipoinflammatory (30, 33). Oleate was chosen as a nonlipotoxic NEFA (33, 34) present in human plasma at concentrations comparable to those of palmitate. Again, mRNA expressions of *FABP3*, *IL6*, *IL8*, *PLIN2*, and *TLR2* were selected for analysis. Increased mRNA expressions of *IL6* ($P = 0.008$), *IL8* ($P = 0.003$), *PLIN2* ($P = 0.01$), and *TLR2* ($P = 0.01$) were found after stimulation with palmitate [Fig. 4(f)], whereas *FABP3* mRNA was unchanged ($P = 0.5$). Statistical differences in mRNA expression were also present on the protein level. Palmitate stimulation of trophoblasts resulted in a statistical trend for higher IL8 concentration in cell culture supernatant ($P = 0.09$) [Fig. 4(g)]. In contrast, stimulation of trophoblasts with oleate resulted in no differential expression ($P > 0.05$). IL1B levels in the cell culture supernatant were below the detection limit. Primary trophoblasts of women with NGT and GDM did not respond significantly differently to NEFA stimulation.

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

The number of villus macrophages was quantified to determine whether NEFA-mediated changes in trophoblastic chemokine and cytokine release have a biological consequence within the placenta. Although CD68 staining within the villus stroma was weak in placentas from women with NGT [Fig. 5(a)], it was stronger in GDM pregnancies [Fig. 5(b)]. A statistically significant elevation of CD68-positive villi was observed within the placenta of women with GDM ($P = 0.02$) [Fig. 5(c)].

## Cord blood lipid profiles and cytokine pattern

To assess potential consequences of placental lipoinflammation for fetal metabolism, we determined

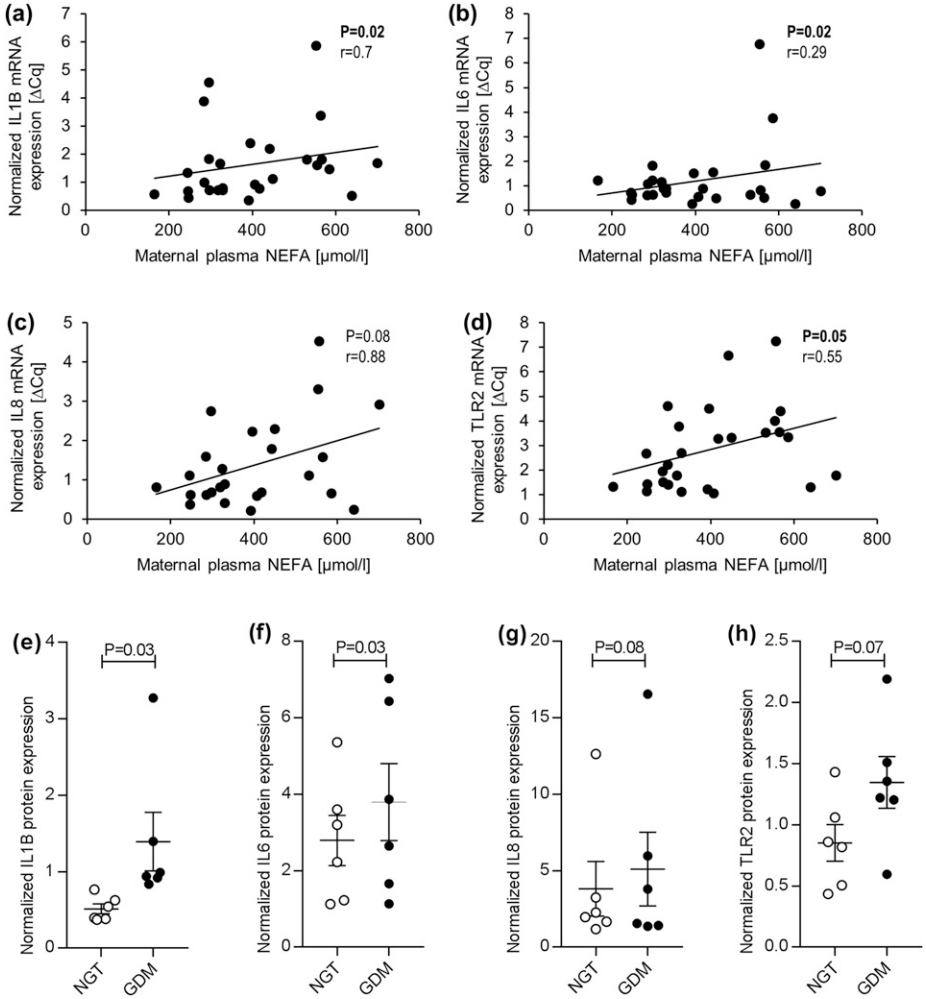


**Figure 3.** Gene expression in whole-placenta tissue. Correlation of maternal plasma NEFAs with placental mRNA expression of (a) IL1B, (b) IL6, (c) IL8, and (d) TLR2. Correlation value (*r*) and *P* value are shown. Protein expression of (e) IL1B, (f) IL6, (g) IL8, and (h) TLR2 in the placental tissue of women with NGT (N = 6; white dots) and women with GDM (N = 6; black dots) with similar BMI. Individual mean ± SEM data are shown. Significant differences ($P \leq 0.05$) of multiple linear regression analyses are shown in boldface.

inflammatory patterns and NEFA profiles of neonates of women with GDM and women with NGT and a similar BMI. Inflammatory parameters in cord blood of neonates of women with GDM and NGT were quantified in the PEACHES cohort. Significantly reduced concentrations in cord blood samples of women with GDM were found for eotaxin ($P = 0.05$), FGF-2 ($P = 0.05$), IL4 ($P = 0.006$), IL9 ($P = 0.02$), IL17 ($P = 0.004$), IP10 ($P = 0.04$), MIP1$\beta$ ($P = 0.01$), and TNF$\alpha$ ($P = 0.02$) [Fig. 4(d)]. In groups with smaller sample sizes (concentrations outside the standard curve), GM-CSF ($P = 0.02$), IL6 ($P = 0.02$), and IL15 ($P = 0.02$) levels were reduced (data not shown). No changes were seen for IL1ra, MCP1, and PDGF, as well as for granulocyte-colony stimulating factor, IFN-$\gamma$, IL8, MIP1$\alpha$, regulated on activation, and VEGF (data not shown, $P > 0.1$). Insulin therapy was not associated with cord blood cytokines among women positive for GDM (data not shown; $P > 0.1$). Even though there was no GDM-related increase in cord blood insulin level, significant positive associations ($P < 0.05$) between cord blood insulin levels and cord blood inflammatory parameters (eotaxin, FGF, IFN-$\gamma$, IL1ra, IL4, IL9, IL17, IP10, MIP1$\alpha$, MIP1$\beta$, TNF$\alpha$, and VEGF) were observed in the overall analyzed PEACHES cohort. Furthermore, statistical trends for reduced levels of SFA C18:0, C20:0, and C24:0 were detectable in the NEFA fraction in cord blood of GDM offspring in comparison with controls ($P = 0.09$ for all) [Fig. 5(c) and 5(f); Supplemental Fig. 2(c)].

## Discussion

The increasing incidences of adiposity and T2D have been discussed as a consequence of adverse fetal programming during pregnancy (4). GDM-associated changes in maternal and placental lipid metabolism may modify the fetal risk of insulin resistance *in utero*. However, the direct influences of maternal lipids on placental and, by extension, fetal lipid

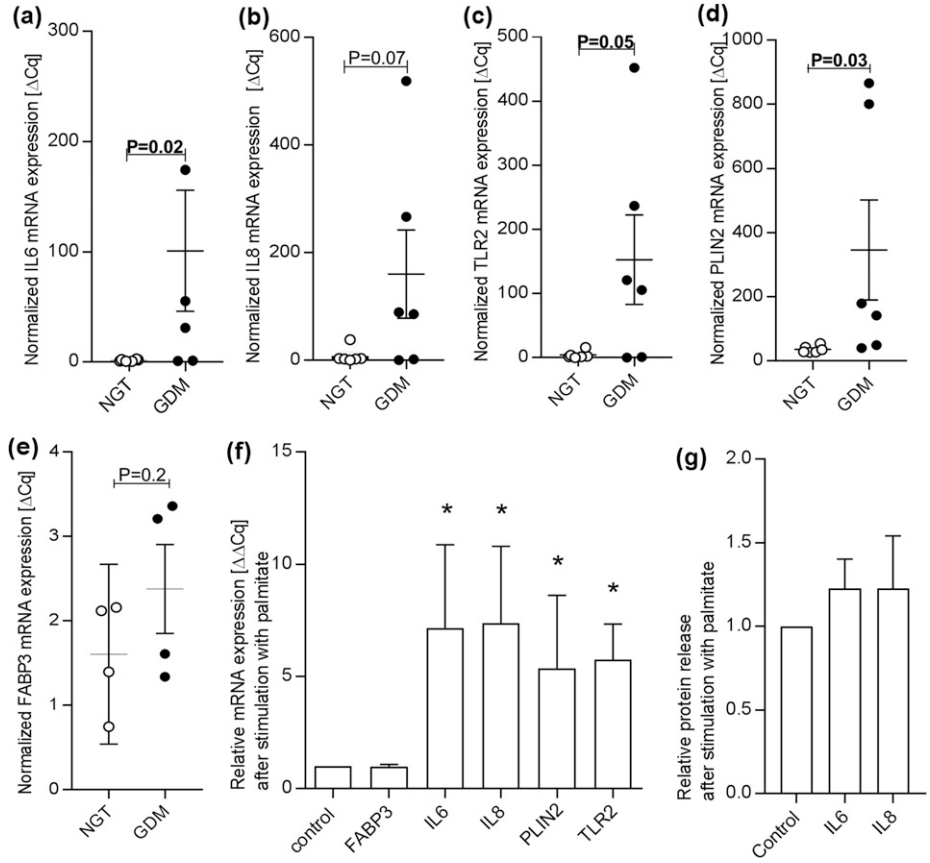


**Figure 4.** Expression of inflammatory genes in trophoblasts. mRNA expression of (a) IL6, (b) IL8, (c) TLR2, (d) PLIN2, and (e) FABP3 in primary trophoblasts of NGT (white dots) and GDM (black dots) women (N ≥ 8) after 48 hours' incubation with medium only. A one-tailed Student *t* test was applied. (f) mRNA expression of primary trophoblasts (N = 14) after 24-h stimulation with 100 μM of palmitate. (g) Amounts of IL6 and IL8 in the supernatant of primary trophoblasts after 24-h stimulation with 100 μM of palmitate. Expression or release is given as FC with respect to the BSA control. Means ± SEM are shown. Significant changes ($P \leq 0.05$) are indicated in boldface or with an asterisk and show the results of ANOVA followed by a Dunnett test.

metabolism are currently not understood and need to be identified.

As an easily accessible tissue at the interface of maternal and fetal metabolism, the placenta is of high relevance for gaining insight into fetal metabolism (35). Increased expression of genes involved in inflammation (15, 16, 36, 37) and lipid metabolism (13, 14, 29, 38) in the placental tissue of obese pregnant women and women with GDM (not independent of BMI) may be relevant for fetal programming. One major limitation of the currently available studies focusing on placental changes in GDM is that they do not correct for the influence of maternal BMI. Therefore, it is not clear whether placental changes described in these studies are an effect of elevated maternal BMI or maternal GDM. Epidemiological data propose an add-on effect of GDM in adverse fetal programming (4, 17). An improved understanding of the causes and underlying mechanisms contributing to a potential add-on effect may provide new strategies for the prevention of adverse fetal outcome in GDM.

To investigate whether BMI-independent but GDM-associated changes in the placenta contribute to adverse fetal outcome such as insulin resistance (39, 40), we analyzed maternal plasma and placental lipid profiles and their effects on placental lipoinflammation. We observed significant elevations of nonesterified SFAs in plasma and total TG in the placental tissue of women with GDM. This elevation in TGs is in accordance with earlier results (11, 41). Similar to earlier studies (15, 16), we identified elevated mRNA and protein expressions of proinflammatory cytokines (IL6 and IL1B) and TLR2 in the whole-placenta tissue of women with GDM. We showed similar findings in primary trophoblasts cultured in medium for 48 hours without any manipulations. These findings could result from sustained FA-induced effects on gene expression, with epigenetics being a possible mechanism (42). Stimulation with palmitate but not oleate was associated with elevated mRNA and protein expressions of IL6 and IL8. The specific mechanisms are unknown, but TLR signaling has been implicated in the effects of SFAs on cellular release of cytokines (IL6, IL1B, TNFα) and chemokines (IL8) (42). Similar responses to increased levels of SFA have been found [*e.g.,* in the hypothalamus) (10, 43), skeletal

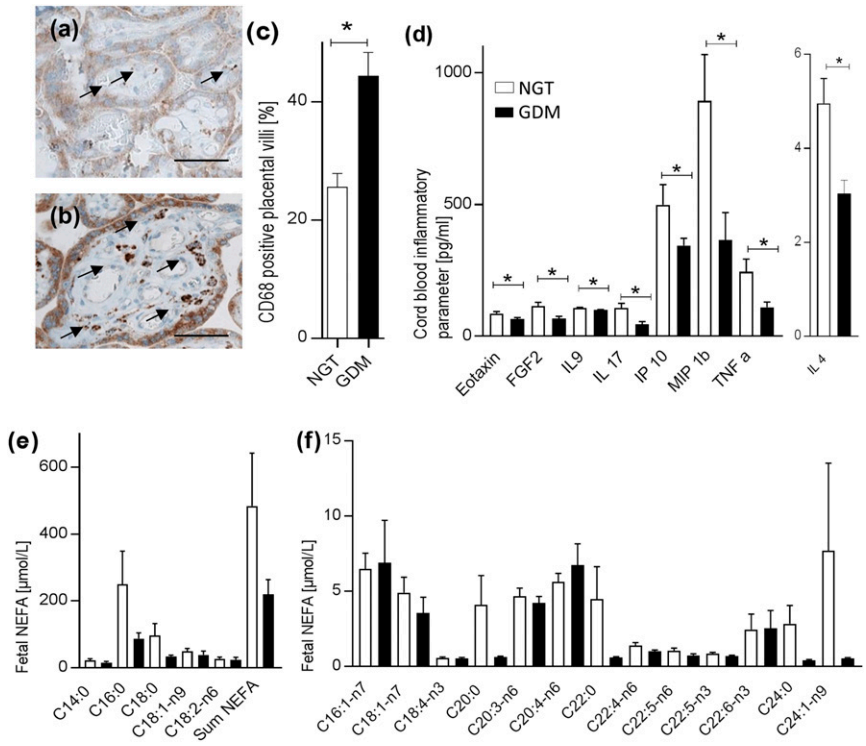


**Figure 5.** Biological relevance of placental lipoinflammation. Staining of placental tissue derived from (a) a woman with NGT and (b) a woman with GDM with antibodies against CD68 (brown). Arrows indicate CD68-positive cells. Representative images; bar = 50 μm. (c) Percentage of CD68-positive villi in the tissue of women with GDM (N = 5) and NGT controls (N = 5) with similar BMI. (d) Concentration of significantly different inflammatory parameters in cord blood samples of women with GDM (N = 52) and NGT controls (N = 52) with similar BMI. Cord blood concentrations of (e) highly abundant (>10 μmol/L) and (f) low abundance (<10 μmol/L) NEFA patterns in cord blood samples of women with GDM [N = 7 (black)] and NGT controls [N = 11 (white)]. Individual mean ± SEM data are shown. Multiple linear regression analyses were conducted. Statistically significant differences ($P \leq 0.05$) are marked with an asterisk.

muscle cells (33), adipocytes (44), and pancreatic islets (45)]. All these findings were triggered by increased SFA levels. In this study, we showed that SFAs are also elevated in maternal plasma and placental tissue of women with GDM. In addition to TLR-signaling, NEFA-mediated placental cytokine/chemokine expression may also be mediated by free FA receptors (46) or peroxisome proliferator-activated receptors. All three mechanisms are active in placental tissue (47) and trophoblasts (36, 38, 48, 49).

Increased expression of IL8 was reported to attract macrophages (50) and therefore may be a potential mediator of placental macrophage infiltration and enhanced placental lipoinflammation. Indeed, we found more CD68-positive macrophages in the placental tissue of women with GDM than in BMI-matched women with NGT. Because we could not detect any GDM-associated elevations of inflammatory parameters and SFAs in fetal cord blood, we propose that GDM-associated placental accumulation of SFAs and lipoinflammation are not directly transmitted to the fetal circulation and are therefore unlikely to be involved with the *in utero* development of adverse fetal outcomes. This suggests a protective role of the placenta for the developing fetus, at least in pregnancies with well-controlled GDM at the end of the pregnancy. Future studies need to investigate whether GDM is associated with changes in the placental metabolome profile. Furthermore, epigenetic studies should be performed to investigate mechanisms for basal mRNA expression differences found in the trophoblasts of women with NGT and GDM.

We acknowledge some limitations of the study. Our findings may not reflect the overall placental lipid and gene profiles as a whole, as we collected placental samples only from the fetal-facing surface of the placenta in low distance to the umbilical cord insertion. Furthermore, we cannot exclude influences of dietary differences between women with NGT and women with GDM in our data because no dietary information during pregnancy was collected in the study. The limited sample sizes available in defined subexperiments are another limitation of this study. Furthermore, maternal BMI and, in part, diagnostic criteria (22, 23) were not identical because independent cohorts were used in different subexperiments. Although we corrected for maternal BMI, different clinical characteristics and treatment strategies within the independent cohorts may have influenced the results.

## Conclusion

Based on our findings and previous reports, we propose that maternal GDM confers a BMI-independent add-on effect on maternal plasma and placental changes in NEFA profiles and FA-mediated activation of lipoinflammatory pathways in trophoblasts. However, neither inflammatory parameters nor SFAs appear to be transmitted to the fetus, and the placenta appears to protect the fetus from these metabolically adverse factors.

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