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Original Paper

Fetal Serum Metabolites Are Independently Associated with Gestational Diabetes Mellitus

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Key Words
Gestational diabetes • Metabolomics • Phosphatidylcholine acyl-alkyl C 32:1 • Proline

Abstract
Background/Aims: Gestational diabetes (GDM) might be associated with alterations in the metabolomic profile of affected mothers and their offspring. Until now, there is a paucity of studies that investigated both, the maternal and the fetal serum metabolome in the setting of GDM. Mounting evidence suggests that the fetus is not just passively affected by gestational disease but might play an active role in it. Metabolomic studies performed in maternal blood and fetal cord blood could help to better discern distinct fetal from maternal disease interactions. ***Methods:*** At the time of birth, serum samples from mothers and newborns (cord blood samples) were collected and screened for 163 metabolites utilizing tandem mass spectrometry. The cohort consisted of 412 mother/child pairs, including 31 cases of maternal GDM. ***Results:*** An initial non-adjusted analysis showed that eight metabolites in the maternal blood and 54 metabolites in the cord blood were associated with GDM. After Benjamini-Hochberg (BH) procedure and adjustment for confounding factors for GDM, fetal phosphatidylcholine acyl-alkyl C 32:1 and proline still showed an independent association with GDM. ***Conclusions:*** This study found metabolites in cord blood which were associated with GDM, even after adjustment for established risk factors of GDM. To the best of our knowledge, this is the first study demonstrating an independent association between fetal

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serum metabolites and maternal GDM. Our findings might suggest a potential effect of the fetal metabolome on maternal GDM.

Introduction

GDM is defined as any degree of glucose intolerance with onset or first recognition during pregnancy. Commonly, the diagnosis is based on results from an oral glucose tolerance test at 24–28 weeks of gestation [1]. GDM is one of the most common complications of pregnancy and its prevalence is constantly rising [2]. If uncontrolled, GDM results in overt hyperglycemia which may significantly increase perinatal morbidity and mortality [3]. Women with GDM have a higher risk of preeclampsia and cesarean section, [4, 5] whereas complications for their newborns include a higher risk for macrosomia [5-7] and fetal hypoglycemia [4, 8]. Potential long-term consequences for the health of mother [9-11] and child [10, 12, 13] may be an impaired glucose tolerance, obesity, and metabolic disorders. Even though GDM usually resolves after birth and blood glucose returns to normal levels, mothers that developed GDM during pregnancy have an increased risk for type 2 diabetes mellitus (T2DM) [14]. Therefore, screening and treatment for GDM are common in most developed countries. Randomized controlled trials have shown improved maternal and neonatal outcomes for these strategies [15,]. But even with strict glycaemic control GDM still represents a risk for adverse pregnancy outcomes. It is known that ethnicity, higher maternal age, obesity, greater weight gain during pregnancy, and hypertension display risk factors for GDM [17].

The pathogenesis of GDM is multifactorial and exact mechanisms underlying the development of the disease are still poorly understood. A traditional pathophysiologic concept proposes that pancreatic β-cells are not able to account for the physiologic pregnancy-related decline in tissue sensitivity to insulin. Glucose intolerance occurs as a result of an inadequate increase in insulin secretion [18]. The placenta secretes cytokines and other factors which add to pregnancy-induced insulin resistance [19]. Other potentially contributing factors discussed in the literature include chronic low-grade inflammation [20], different genetic, epigenetic and non-genetic environmental factors including nutrition [21-26]. Moreover, fetal sex [23, 27] and fetal genes [28] have been shown to correlate with maternal glucose concentrations during pregnancy and thus may modulate the risk for maternal GDM. However, it is not clear if the fetus can impact on the maternal organism in such a regulating manner. Pathophysiologic pathways of development and progression of GDM still need to be investigated more thoroughly in order to better understand a potential involvement of a fetal influence.

Metabolomics is an investigative approach that analyses products of biochemical pathways in a detailed way [29]. It is a robust, rapid, and efficient method to analyze a large number of small molecules in tissues, urine, blood and other biological fluids. This approach is well suited to find biomarkers for the prediction, diagnosis, and monitoring of several diseases including metabolic disorders like GDM [30]. It can also help to better understand physiologic and pathophysiologic processes on a molecular level and, as such, in a more detailed manner. However, the knowledge of the human metabolome in general still presents a big challenge to science. This is especially true for a period like pregnancy where the body undergoes multiple physiologic changes.

In this study, we wanted to investigate characteristic disease-associated metabolites in the serum of pregnant women with GDM and compare them to the findings from women without GDM. Moreover, as there is a lack of studies that investigated the fetal metabolome in GDM, we compared the metabolic cord blood profile of newborns from mothers with GDM to the profile of newborns from mothers without GDM.

Materials and Methods

Clinical study

This observational study was approved by the local Ethics Committee. A total of 412 pregnant women who delivered their newborns at the Charité obstetrics department in Berlin, Germany were invited to participate (Berlin Birth Cohort, ref: [31, 32]). As the focus of this study was set on patients with GDM, mothers with overt diabetes before pregnancy were not included. The majority of mothers (n = 344) were of European background, the others had an African, Asian, or Arabic background.

After written consent was obtained, a structured medical history was taken. The following data were extracted into our database: age, ethnicity, body height and body weight before pregnancy, diabetes mellitus, and hypertension during pregnancy, smoking during pregnancy, systolic and diastolic blood pressure (BP) measurements recorded during pregnancy, and mode of delivery. Biometric data of the newborns were collected during the routine postnatal examination: birth weight, birth length, ponderal index (birth length (m) / the cube root of weight (kg)), head circumference, child sex, and Apgar score 5 minutes postnatally and Apgar score 10 minutes postnatally were screened and assessed [33]. Gestational age at delivery was based on the last menstrual period and anamnestically assessed during the first pregnancy examination. Midwives collected maternal blood from the cubital vein in the delivery room or on the ward. Fetal blood samples were collected from the umbilical cord within 10 min after delivery. Blood was centrifuged at 2750 g immediately after its withdrawal and the obtained serum was stored at -80 °C until measurements were performed. Obtained serum samples were used for metabolomic analyses and additionally to measure glucose and insulin concentrations. GDM was screened and assessed according to the practice guideline of the German Diabetes Association (DDG) and the German Association for Gynecology and Obstetrics (DGGG) [34]. In total, 31 out of 412 pregnant women were diagnosed with GDM.

Targeted metabolomics in maternal and fetal blood samples

The targeted metabolomics approach was based on flow injection analysis–electrospray ionization–tandem mass spectrometry (FIA–ESI–MS/MS) measurements with the Absolute IDQTM p150 kit (BIOCRATES Life Sciences AG, Innsbruck, Austria). For more details of the assay workflow see Fig. 1. The assay allows simultaneous quantification of 163 metabolites out of 10 µL serum and includes free carnitine, 40 acylcarnitines (Cx:y), 14 amino acids (13 proteinogenic + ornithine), hexoses (sum of hexoses – about 90-95 % glucose), 92 glycerophospholipids (15 lysophosphatidylcholines (lysoPC) and 77 phosphatidylcholines (PC)), and 15 sphingolipids (SMx:y). The abbreviations Cx:y are used to describe the total number of carbons and double bonds of all chains, respectively. The method of the Absolute*IDQ*™ p150 kit has been proven to be in conformance with the FDA-Guidlines "Guidance for Industry - Bioanalytical Method Validation (May 2001)" [35], which implies proof of reproducibility within a given error range. Measurements were

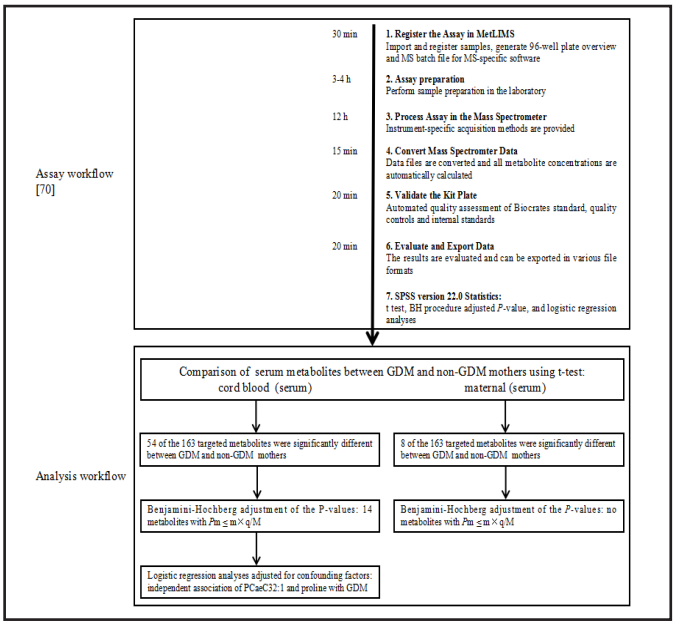

Fig. 1. Flowchart for the metabolomics analysis strategy. M = total number of analyzed metabolites (M=163); q = FDR; m = the individual rank of tested metabolite; Pm = the individual P-value; PC = phosphatidylcholine; ae = acyl-alkyl. The Assay Workflow was adapted from: AbsoluteIDQ® p150 Kit - Biocrates. Pge 2. Assay Workflow. http://www.biocrates.com/images/p150_KitFolder.pdf.

performed as described in the manufacturer's manual. This manual contains comprehensive instructions and detailed information on analytical specifications, including the limit of detection (LOD), specificity, accuracy, reproducibility, and all other specifications. The assay procedures of the Absolute *IDQ*™ p150 kit, as well as the metabolite nomenclature, have been described in detail previously [36, 37]. Sample handling was performed by a Hamilton Microlab STAR™ robot (Hamilton Bonaduz AG, Bonaduz, Switzerland) and a Ultravap nitrogen evaporator (Porvair Sciences, Leatherhead, U.K.). Mass spectrometric analyses were done on an API 4000 triple quadrupole system (Sciex Deutschland GmbH, Darmstadt, Germany) equipped with a 1200 Series HPLC (Agilent Technologies Deutschland GmbH, Böblingen, Germany) and a HTC PAL auto sampler (CTC Analytics, Zwingen, Switzerland) controlled by the software Analyst 1.6.1. Data evaluation for quantification of metabolite concentrations and quality assessment was performed with the MetIDQ™ software package, which is an integral part of the Absolute*IDQ*™ kit. Internal standards served as a reference for the calculation of metabolite concentrations [μM].

Statistical analysis

Data were analyzed with SPSS version 22.0. Results of quantitative data were expressed as the arithmetic mean ± standard deviation (SD). An unpaired t-test was used for comparison of continuous variables between two groups. To reduce false discovery rate (FDR) after t-test, *P*-values were adjusted using the Benjamini-Hochberg procedure. The BH procedure is defined as $P_m \leq m \times q/M$ [38, 39]. M = total number of tested metabolites (M=163), q = FDR (the FDR set up at 5% in the present paper), *P*m = the individual *P*-value's rank, m = the individual rank of the tested metabolite. Pearson's chi-square test was used for testing qualitative data. In the next step, we performed logistic regression analyses to correct for known confounding factors. Relevant confounding factors of GDM mentioned in current literature were included into the models: maternal age, pre-pregnancy body mass index (BMI), ethnicity, family history of diabetes, and smoking during pregnancy. A flowchart for the metabolomics analysis strategy is given in Fig. 1. A *P*-value less than 0.05 was considered significant.

Results

Description of the cohort

Descriptive data of the study population are given in Table 1. The study population represented a typical German birth cohort in regards to key characteristics like maternal age, ethnicity, BMI before pregnancy, gravidity, parity, and biometric data of the newborns (for more details, see Table 1).

Table 1. Detailed descriptive data of all mother/child pairs (n = 412). Data are given as mean ± SD or %

Variable	Mean±SD / %
Maternal age, y	30.5±5.9
Maternal height, cm	166.3±7.2
Maternal BMI before pregnancy, kg/m²	22.6±4.5
Smoking before pregnancy, %	40.8
Smoking during pregnancy, %	14.6
Hypertension before pregnancy, %	3.40
Hypertension during pregnancy, %	9.5
Diabetes during pregnancy, %	8.1
Mean systolic BP 3rd trimester of pregnancy, mm Hg	114.0±9.8
Mean diastolic BP 3rd trimester of pregnancy, mm Hg	69.6±6.9
Gestational age at delivery, day	271.7±11.4
Child sex, male/female, %	50.8/49.2
Child birth weight, g	3346.9±581.9
Child birth length, cm	50.7±2.7
Ponderal index	25.6±2.3
Head circumference, cm	34.7±1.5
Apgar score at 5 min	9.3±1.0
Apgar score at 10 min	9.5±0.9

Pregnancy outcomes of mothers and newborns

Mothers with GDM had a significantly higher age compared to non-GDM mothers. There were no significant differences in blood glucose levels or in any of the other recorded parameters between the two groups (for more details, see Table 2).

Newborns from mothers with GDM had a significantly higher preterm birth rate compared to newborns from non-GDM mothers. Crucial parameters of fetal outcome like birth weight or ponderal index were not

Table 2. Descriptive data of mothers grouped according to GDM (n = 412). Data are given as mean ± SD or %

Variable	Non-GDM (n =381)	GDM (n = 31)	χ^2 /t	P value
Maternal age, y	30.3±5.9	32.6±6.2	-2.01	0.045
Maternal height, cm	166.4±7.0	165.4±8.9	0.79	0.431
Pre-pregnancy BMI, kg/m²	22.5±4.4	24.8±6.5	2.00	0.054
Ethnicity, n (%)				
Caucasian	316 (84.9%)	28 (90.3%)	0.66	0.416
Other	56 (15.1%)	3 (9.7%)		
Weight gain during pregnancy, kg	13.1±7.2	13.7±8.2	-0.38	0.704
Gestational hypertension, n (%)	36 (9.5%)	3 (9.7%)	0.001	0.974
Smoking during pregnancy, n (%)	54 (14.2%)	6 (19.4%)	0.27	0.606
C-section, n (%)	22 (5.8%)	2 (6.4%)	0.51	0.612
Maternal glucose, mmol/L	5.2±1.4	5.2±1.3	0.13	0.899
Maternal insulin, mIU/L	37.2±36.9	29.6±22.8	0.74	0.460

significantly different in newborns from mothers with GDM when compared to newborns from non-GDM mothers (for more details, see Table 3).

Results from targeted metabolomics in maternal and newborn blood samples

In total, eight of the 163 targeted maternal serum metabolites differed significantly between mothers with GDM and mothers without GDM (1 acylcarnitines, 2 diacyl-PCs, 4 acyl-alkyl-PCs, and sum of hexoses.). After adjusting the *P*-values using the Benjamini–Hochberg procedure no metabolites were significantly different between mothers with GDM and mothers without GDM (for more details, see Table 4).

In fetal cord blood, 54 of the 163 targeted metabolites were significantly different in newborns of mothers with GDM compared to non-GDM mothers. These metabolites included 10 amino acids, 5 sphingomyelins, 1 hydroxy-sphingomyelin, and 38 glycerophospholipids (for more details, see Table 5). After BH adjustment of the *P*-values fourteen metabolites (3 amino acids, 2 sphingomyelins, and 9 glycerophospholipids (2 lyso-PCs, 4 diacyl-PCs, and 3 acyl-alkyl-PCs)) with $P_m \leq m \times q/M$ remained significantly different in fetal cord blood from GDM mothers compared to non-GDM mothers (for more details see Table 5). Furthermore, Manhattan Plot of all fetal serum metabolites is given in Fig. 2.

Table 3. Descriptive data of newborn grouped according to maternal GDM (n = 412). Data are given as mean ± SD or %

Variable	Non-GDM (n = 381)	GDM (n = 31)	χ^2/t	P value
Gestational age, (d)	272.2±11.3	265.8±10.6	3.05	0.002
Gestational age, n (%)				
< 259 d	20(5.5%)	4(12.9%)		
259～280 d	293(81.2%)	27(87.1%)		
> 280 d	48(13.3%)	0(0.0%)	6.77	0.034
Birth weight (g)	3352.3±514.7	3297.1±575.8	0.57	0.285
Birth weight, n (%)				
< 2500 g	17(4.6%)	3(9.7%)		
2500～4000 g	316(84.7%)	27(87.1%)		
> 4000 g	40(10.7%)	1(3.2%)	3.12	0.210
Birth length, cm	50.7±2.7	50.2±3.2	1.07	0.285
Head circumference, cm	34.7±1.5	34.2±1.6	1.90	0.066
Ponderal index	25.6±2.9	26.1±2.8	-1.31	0.191
Apgar score				
5 min	9.3±1.0	9.2±1.0	0.48	0.631
10 min	9.6±0.9	9.5±0.8	0.41	0.684
Fetal glucose, mmol/L	2.9±1.3	3.0±2.6	-0.10	0.924
Fetal insulin, mIU/L	6.8±3.9	9.6±10.4	-0.59	0.585

Table 4. Comparison of maternal serum metabolites between non-GDM and GDM. Data are given as mean ± SD. Cx:y=acylcarnitines, the abbreviations Cx:y are used to describe the total number of carbons and double bonds of all chains, respectively; PC = phosphatidylcholine; a = acyl; aa = diacyl; ae = acyl-alkyl; SM = Sphingomyelins; H1 = sum of hexoses. Note. Only metabolites with $P_m \leq 0.05$ were shown in the table. M = total number of analyzed metabolites (M=163); q = FDR; m = the individual rank of tested metabolite; Pm = the individual P-value

Variable	Non-GDM (n = 381) μM	GDM (n = 31) μM	t	P_m value	m×q/M value
Acylcarnitines and Hydroxy-&dicarboxy-acylcarnitines					
C4:1	$1.44\times10^{-2}\pm3.73\times10^{-3}$	$1.64\times10^{-2}\pm4.69\times10^{-3}$	-2.15	0.033	9.20×10^{-4}
Diacyl-phosphatidyl-cholines					
PC aa C36:5	19.60±9.57	24.91±11.83	-2.28	0.024	6.10×10^{-4}
PC aa C36:6	1.68±0.65	2.01±0.85	-2.05	0.041	1.84×10^{-3}
Acyl-alkyl-phosphatidyl-cholines					
PC ae C38:0	3.54±1.23	4.18±1.59	-2.10	0.037	1.23×10^{-3}
PC ae C38:3	17.06±5.33	19.66±6.16	-2.01	0.046	2.45×10^{-3}
PC ae C40:1	3.94±1.56	4.74±2.33	-2.03	0.044	2.15×10^{-3}
PC ae C40:5	8.79±2.69	10.16±3.26	-2.09	0.038	1.53×10^{-3}
Sum of hexoses					
H1	4565.46±1679.64	6346.35±2585.17	-2.95	0.008	3.07×10^{-4}

Logistic regression

Following the univariate analyses, logistic regression models were calculated to see which of the identified fetal metabolites were independently associated with GDM. Logistic regression models were adjusted for age, ethnicity, family history of diabetes, and pre-pregnancy BMI, and smoking during pregnancy, all known risk factors for GDM [19, 40]. An additional model was calculated including gestational age, as GDM offspring had a significantly reduced gestational age and it is know that gestational age is a contributing factor to the fetal and maternal metabolome [41, 42].

All these models demonstrated an independent association of the phosphatidylcholine acyl-alkyl C 32:1 (PC ae C 32:1) and the amino acid proline with GDM (for more details, see Table 6).

PC ae C 32:1, proline, GDM and preterm birth

As mentioned above, GDM offspring displayed a significantly reduced gestational age. Adding gestational age as a confounder in logistic regression analysis (Model D) did not affect the independent association between fetal PC ae C 32:1, proline, and GDM. To get further

insight if these metabolites might be associated with a reduced gestational age in GDM we calculated two additional multivariable regression models (Table 7). Model A consisted of known factors affecting gestational age at birth, including pre-pregnancy BMI, history of preterm birth, smoking during pregnancy and the maternal GDM status. In Model B fetal concentrations of PC ae C 32:1 and proline were added. Model A showed a significant association between gestational age and GDM status of the mother and history of preterm birth. Adding fetal PC ae C 32:1 and proline in Model B rendered the previously significant association between GDM and gestational age insignificant, yet both metabolites demonstrated a significant association with gestational age. This result indicates that altered levels of PC ae C 32:1 and proline in cord blood of GDM offspring might impact on GDM related reductions in gestational age at birth.

Discussion

In the current study, 163 metabolites were analyzed in maternal and fetal cord blood of 412 delivering women. 31 of the participating pregnant women had been diagnosed with GDM. The goal of the study was to identify associations between

Table 5. Comparison of newborn serum metabolites between non-GDM and GDM. Data are given as mean ± SD. Data are given as mean ± SD. PC = phosphatidylcholine; a = acyl; LPC = lysophosphatidylcholine; aa = diacyl; ae = acyl-alkyl; SM = Sphingomyelins; OH = hydroxy. Note. Only metabolites with $P_m \leq 0.05$ were shown in the table. M = total number of analyzed metabolites (M=163); q = FDR; m = the individual rank of tested metabolite; Pm = the individual P-value

Variable	Non-GDM (n = 381) µM	GDM (n = 31) µM	t	P_m value	m×q/M value
Amino acids					
Gln	398.04±106.67	449.75±120.24	-2.57	0.011	0.010
His	111.08±28.66	128.02±27.28	-3.18	1.60×10^{-3}	9.20×10^{-4}
Met	36.49±10.06	41.39±7.27	-2.66	8.24×10^{-3}	7.67×10^{-3}
Phe	69.33±17.97	76.08±14.10	-2.04	0.042	0.016
Pro	182.78±47.68	219.47±51.19	-4.10	5.00×10^{-5}	3.07×10^{-4}
Ser	136.62±43.47	152.85±38.93	-2.01	0.045	0.017
Thr	217.31±81.80	248.62±61.74	-2.08	0.038	0.015
Tyr	73.00±20.86	84.04±17.37	-2.87	4.35×10^{-3}	5.21×10^{-3}
Val	187.19±55.22	215.66±50.06	-2.78	5.71×10^{-3}	6.44×10^{-3}
xLeu	206.33±56.82	233.94±63.44	-2.58	0.010	9.20×10^{-3}
Lyso-phosphatidyl-colines					
LPC 14:0	3.80±0.42	3.96±0.40	-2.07	0.039	0.015
LPC 16:0	53.53±15.96	59.99±11.28	-2.21	0.028	0.014
LPC 16:1	3.72±1.38	4.42±1.33	-2.72	6.85×10^{-3}	7.06×10^{-3}
LPC 18:1	12.29±4.24	14.54±3.50	-2.87	4.30×10^{-3}	4.60×10^{-3}
LPC 18:2	11.49±4.56	13.49±3.05	-3.35	1.63×10^{-3}	1.23×10^{-3}
LPC 20:3	3.95±1.45	4.58±1.28	-2.36	0.019	0.013
LPC 20:4	13.53±5.15	16.00±4.71	-2.58	0.010	9.51×10^{-3}
Diacyl-phosphatidyl-cholines					
PC aa C28:1	0.93±0.36	1.08±0.50	-2.20	0.029	0.014
PC aa C30:0	4.12±1.25	4.78±0.92	-2.91	3.81×10^{-3}	3.68×10^{-3}
PC aa C32:0	14.73±4.78	17.28±3.63	-2.91	3.86×10^{-3}	3.99×10^{-3}
PC aa C32:1	11.92±4.62	14.48±4.03	-3.00	2.91×10^{-3}	2.45×10^{-3}
PC aa C32:2	1.02±0.52	1.23±0.86	-2.03	0.043	0.016
PC aa C34:1	104.66±26.66	119.59±27.78	-2.99	3.00×10^{-3}	3.07×10^{-3}
PC aa C34:2	85.16±31.22	101.08±42.55	-2.65	8.41×10^{-3}	7.98×10^{-3}
PC aa C34:4	0.49±0.17	0.60±0.32	-3.00	2.88×10^{-3}	2.15×10^{-3}
PC aa C36:1	24.84±7.17	28.74±7.30	-2.90	3.89×10^{-3}	4.29×10^{-3}
PC aa C36:2	51.05±19.72	61.37±28.65	-2.69	7.35×10^{-3}	7.36×10^{-3}
PC aa C36:3	65.01±19.73	75.68±22.49	-2.86	4.43×10^{-3}	5.52×10^{-3}
PC aa C36:4	123.24±28.67	136.94±25.58	-2.58	0.010	8.90×10^{-3}
PC aa C36:5	5.18±2.46	6.64±2.54	-3.16	1.69×10^{-3}	1.53×10^{-3}
PC aa C36:6	0.32±0.14	0.39±0.18	-2.530	0.012	0.011
PC aa C38:3	41.35±11.77	45.84±9.39	-2.07	0.039	0.015
PC aa C38:5	21.05±5.82	23.70±8.99	-2.32	0.021	0.013
PC aa C38:6	56.98±18.92	65.43±21.45	-2.37	0.018	0.012
Acyl-alkyl-phosphatidyl-cholines					
PC ae C30:0	0.27±0.08	0.30±0.07	-2.20	0.028	0.013
PC ae C30:1	0.18±0.07	0.21±0.07	-2.48	0.014	0.011
PC ae C32:1	2.40±0.80	2.83±0.67	-2.87	4.31×10^{-3}	4.91×10^{-3}
PC ae C32:2	0.48±0.15	0.55±0.12	-2.47	0.014	0.012
PC ae C34:1	4.75±1.65	5.72±1.74	-3.15	1.77×10^{-3}	1.84×10^{-3}
PC ae C34:2	3.02±1.21	3.62±1.96	-2.49	0.013	0.011
PC ae C36:3	1.99±0.80	2.34±1.33	-2.23	0.026	0.013
PC ae C36:4	8.88±2.80	10.22±2.78	-2.57	0.011	0.010
PC ae C36:5	6.37±2.18	7.34±2.26	-2.37	0.018	0.012
PC ae C38:4	7.59±2.23	8.47±2.37	-2.09	0.037	0.014
PC ae C38:5	7.14±2.25	8.32±2.79	-2.76	6.01×10^{-3}	6.75×10^{-3}
PC ae C38:6	2.96±1.01	3.54±1.25	-2.99	2.93×10^{-3}	2.76×10^{-3}
PC ae C40:0	6.70±0.99	7.19±1.15	-2.60	0.010	8.59×10^{-3}
PC ae C40:1	0.95±0.42	1.05±0.24	-2.11	0.041	1.56×10^{-3}
Hydroxy-sphingomyelins					
SM (OH) C16:1	1.67±0.54	1.93±0.52	-2.61	9.47×10^{-3}	8.28×10^{-3}
Sphingomyelins					
SM C16:0	52.30±14.60	60.03±15.26	-2.83	4.96×10^{-3}	6.13×10^{-3}
SM C16:1	9.82±3.09	11.33±3.28	-2.59	0.010	9.82×10^{-3}
SM C18:0	20.24±5.73	23.79±4.85	-3.35	8.77×10^{-4}	6.10×10^{-4}
SM C18:1	13.05±4.22	15.29±3.89	-2.84	4.68×10^{-3}	5.83×10^{-3}
SM C24:1	30.55±9.08	35.54±9.22	-2.94	3.52×10^{-3}	3.37×10^{-3}

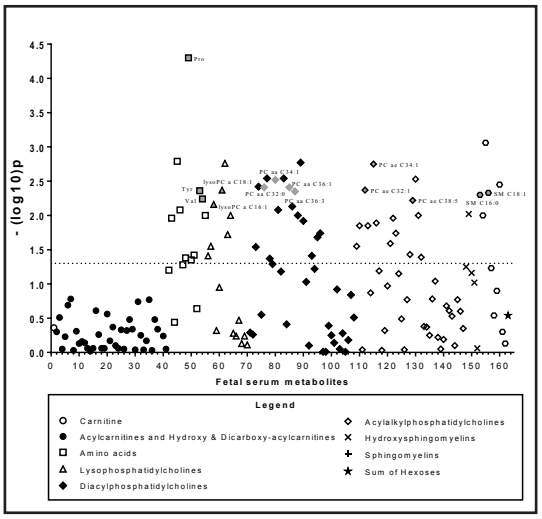

Fig. 2. Manhattan Plot of all fetal serum metabolites. The –log10 of resulted p-values is shown. The level of significance for the unadjusted analyses is shown by a dotted line. Each class of metabolites is marked with a respective symbol. Metabolites shown in grey together with the respective metabolite name indicate metabolites that were still significantly different after Benjamini Hochberg adjustment.

Table 6. Adjusted logistic regression models analyzing associations between newborn metabolites and GDM. PC = phosphatidylcholine; ae = acyl-alkyl. Model A: Considering maternal age, Maternal pre-pregnancy BMI, Ethnicity, and 14 metabolites from the above Table 4. ($P_m \leq m \times q/M$) being the independent variable and GDM being dependent variable. Model B: Model A + family history of diabetes being the independent variable. Model C: Model B + smoking during pregnancy being the independent variable. Model D: Model C + gestational age being the independent variable

Variable	P value	OR	95.0% Confidence interval for B
Model A			
Maternal age	0.051	1.07	1.00～1.15
Maternal pre-pregnancy BMI	0.002	1.11	1.04～1.19
Ethnicity	0.507	1.59	0.40～6.32
Proline	0.005	1.01	1.00～1.02
PC ae C32:1	0.029	1.66	1.06～2.62
Model B			
Maternal age	0.064	1.07	1.00～1.14
Maternal pre-pregnancy BMI	0.002	1.11	1.04～1.19
Ethnicity	0.530	0.80	0.42～1.52
Family history of diabetes	0.965	1.56	0.39～6.23
Proline	0.004	1.01	1.00～1.02
PC ae C32:1	0.029	1.66	1.06～2.62
Model C			
Maternal age	0.063	1.07	1.00～1.14
Maternal pre-pregnancy BMI	0.002	1.11	1.04～1.19
Ethnicity	0.542	1.54	0.38～6.20
Family history of diabetes	0.949	1.03	0.45～2.35
Smoking during pregnancy	0.266	1.81	0.64～5.16
Proline	0.004	1.01	1.00～1.02
PC ae C32:1	0.030	1.65	1.05～2.60
Model D			
Maternal age	0.067	1.07	1.00～1.14
Maternal pre-pregnancy BMI	0.002	1.11	1.04～1.19
Ethnicity	0.459	1.73	0.40～7.42
Family history of diabetes	0.952	1.03	0.45～2.36
Smoking during pregnancy	0.330	1.69	0.59～4.90
Gestational age	0.352	0.98	0.95～1.02
Proline	0.004	1.01	1.00～1.02
PC ae C32:1	0.028	1.66	1.06～2.61

distinctive maternal and fetal metabolites and GDM. Our study identified metabolites in cord blood which were associated with GDM, even after adjustment for established risk factors of GDM. Interestingly, further analyses showed an additional independent interaction between the identified cord blood metabolites and GDM associated reductions in gestational age at birth. To the best of our knowledge,

Table 7. Multiple linear regression models analyzing the association between cord blood PC ae C32:1 and proline and gestational age. PC = phosphatidylcholine; ae = acyl-alkyl. Model A: Considering maternal pre-pregnancy BMI, Maternal GDM, maternal smoking during pregnancy, and history of preterm birth (<37weeks). Model B: Model A + cord blood proline and PC ae C32:1

Variable	Standardized Beta	t	P	95.0% Confidence interval for B
Model A (R^2 = 0.05)				
Maternal pre-pregnancy BMI	-0.03	-0.52	0.605	-0.32～0.19
Maternal GDM	-0.12	-2.29	0.023	-9.16～-0.69
Smoking during pregnancy	-0.03	-0.57	0.568	-4.03～2.22
History of preterm birth	-0.14	-2.63	0.009	-16.42～-2.36
Model B (R^2 = 0.09)				
Proline	-0.15	-2.81	0.005	-0.06～-0.01
PC ae C32:1	-0.12	-2.28	0.023	-3.16～-0.23
Maternal pre-pregnancy BMI	-0.05	-0.99	0.325	-0.38～0.13
Maternal GDM	-0.07	-1.34	0.182	-7.13～1.35
Smoking during pregnancy	-0.03	-0.65	0.518	-4.07～2.05
History of preterm birth (<37weeks)	-0.13	-2.48	0.014	-15.62～-1.79

this is the first study demonstrating an independent association between fetal serum metabolites and maternal GDM. Our findings might suggest a potential effect of the fetal metabolome in maternal GDM.

Analysis of descriptive data of the study population revealed that newborns from mothers with GDM had a significantly higher risk of preterm birth than newborns from non-GDM mothers, in accordance with earlier studies [43, 44]. To investigate, if differences in gestational age might have contributed to the observed differences in PC ae C32:1 and proline cord blood levels, gestational age was added to the logistic regression model. The addition of gestational age did not impact on the independent association between cord blood PC ae C32:1, and proline and GDM. However, two multivariable linear regression models using gestational age as independent variable indicated that the impact of GDM on gestational age might be controlled by cord blood PC ae C32:1 and proline concentrations. This result is in accordance with current literature, at least for proline. Two previous publications also demonstrated a negative correlation between proline, measured in amniotic fluid and neonatal blood, and gestational age [45-48]. To the best of our knowledge, there are no earlier studies that observed an association of proline and PC ae C32:1 with GDM related reductions in gestational age [44].

There was no significant difference in birth weight from newborns from mothers with GDM and newborns of mothers without GDM in our study. This may seem surprising because GDM is a known risk factor for macrosomia of the newborn, at least if untreated [4, 8, 49]. In our study, women with GDM had comparable glucose levels to women without GDM, therefore a good glucose control of mothers with GDM during pregnancy in combination with the reduced duration of gestation might explain the similar birth weight results. Despite comparable glucose levels in both groups, we found differences in the metabolomic profiles of mothers and newborns. Therefore, the observed differences may be independent of blood glucose. This is corroborated by the fact that there was no correlation between the identified metabolites and glucose or insulin (data not shown).

Fetal metabolites associated with maternal GDM

An initial non-adjusted analysis identified 54 metabolites in the cord blood being associated with GDM. After adjusting the *P*-values using the BH procedure and calculating various models corrected for confounding factors of GDM, our study demonstrated that PC ae C32:1 and the amino acid proline were independently associated with GDM.

The traditional pathophysiologic concept states that GDM is a result of environmental cues and maternal genetic predisposition [21, 22]. However, this has currently been challenged by a new theory. It was proposed that fetal genes may also impact on maternal physiology during pregnancy, thus potentially modifying maternal blood pressure and glucose concentration [50]. In women with certain maternal gene polymorphisms, fetal sex influenced important parameters of maternal physiology during pregnancy, including those of the glucose metabolism [23-25]. Moreover, paternally transmitted gene variants of the fetal IGF2 gene (which encodes insulin-like growth factor-II) were associated with increased maternal glucose concentrations, thus potentially altering her risk of developing gestational diabetes mellitus [28].

The mechanisms, however, of how the fetal genotype may influence maternal metabolism are unknown. One hypothesis is that variations in placental function induced by the fetal genome could play a key role in this process, probably by changing the secretion pattern of placental hormones [50, 51]. In fact, the placenta acts as an interphase between mother and child and is partially of fetal origin. Thus, the fetal genome may influence maternal glucose status and blood pressure via placental function in order to guarantee the nutrient supply for the fetus. As such, fetal genes would be able to "demand" an increased flow of nutrients from the maternal blood, in case it is needed, which is best exemplified by multifetal pregnancies. Interestingly, multifetal pregnancies are associated with an increased risk for GDM [52]. A recent meta-analysis including twenty studies and data of over 2, 4 million women showed that pregnant women bearing a male fetus had a 1.04-fold higher risk of developing GDM

than those bearing a female fetus [27]. This data clearly shows that fetal genotype (sex being considered as a genetic variant) is associated with maternal GDM.

Until now it is not known if the fetal metabolome is also associated with maternal GDM. We believe that a comparable concept like one of fetal genes influencing maternal metabolism can be applied to the fetal metabolome. So far, no data has been published explicitly on eventual effects of fetal metabolites on the development of maternal GDM during pregnancy. However, first hints of an association of fetal metabolites and maternal GDM come from other metabolomics studies [53-55].

Up to now, the focus of metabolomics research for GDM lies on analyzing maternal body fluids. Lehman et al. [56] saw decreased levels of plasma diacyl-PCs, lyso-PCs and arachidonic acid being associated with insulin resistance in women with GDM. Liu et al. [57] found changes in serum metabolites of women with GDM, but only in the later stage of pregnancy. Acyl-alkyl-PCs have been associated with T2D in the general population [58]. A European prospective study analyzing more twenty thousand individuals revealed that diacyl-PCs were independently associated with increased risk of T2D and serum acyl-alkyl-PCs were associated with a decreased risk [59]. Importantly, other studies showed that environmental, dietary and lifestyle factors changed metabolic patterns of diacyl-PC and acyl-alkyl-PC in T2DM-cohorts [60-62]. We found distinct fetal acyl-alkyl-PC independently associated with GDM, even after strict adjustment for confounding factors and apparently independent of blood glucose.

Some studies have reported that elevated plasma levels of branched-chain amino acids (BCAAs) also were associated with an increased risk of GDM [63, 64] T2D [64, 65], and insulin resistance [66]. In our present study, there were no significant differences in maternal plasma amino acid levels between GDM and non-GDM mothers. Similarly, Chorell et al. did not observe significant differences in levels of BCAAs during pregnancy, yet were able to demonstrate a significant increase in GDM mothers postpartum [67]. In the current study, ten amino acids displayed higher cord blood concentrations in newborns from mothers with GDM compared to newborns from non-GDM mothers. After BH procedure and strict adjustment for confounding factors for GDM, fetal proline still showed an independent association with GDM. This finding is supported by literature [68]. Cetin et al. [68] also demonstrated higher cord blood levels of the amino acid proline, which was absent in the maternal circulation. Correlation analysis furthermore showed a significant relationship between fetal and maternal proline levels in GDM cases, which could not be found in the absence of GDM. The authors concluded that alterations in placental amino acid exchange and/or fetal/placental amino acid metabolism might have been responsible for this observation.

Our results suggesting a fetal contribution to the development of maternal GDM has to be investigated more thoroughly. Of course, it is also possible that the observed changes in fetal metabolites solely occurred as a consequence to the developing GDM in the mother. We are aware that the idea of a fetal influence on maternal physiology via genes and/or metabolites is a new concept and study results including ours, so far do not give evidence for causality.

Study limitations and Outlook

In this study, serum metabolites were only measured at one occasion prior to birth. Metabolomics data as a reflection of systemic metabolic processes, in general, need to be interpreted with caution as it may be influenced by phenotype and lifestyle factors. However, Floegel et. Al. [69] performed targeted metabolomics at two points in time 4 months apart among 100 healthy subjects and demonstrated that most of the metabolites of a single measurement may be sufficient for risk assessment in epidemiologic studies with healthy subjects.

Despite the mentioned study limitations, findings of the current study may contribute to better understand fetal pathophysiological processes in GDM pregnancies. However, for further conclusions findings of the current study need confirmation in independent prospective studies. Future studies should include collection of information on possible

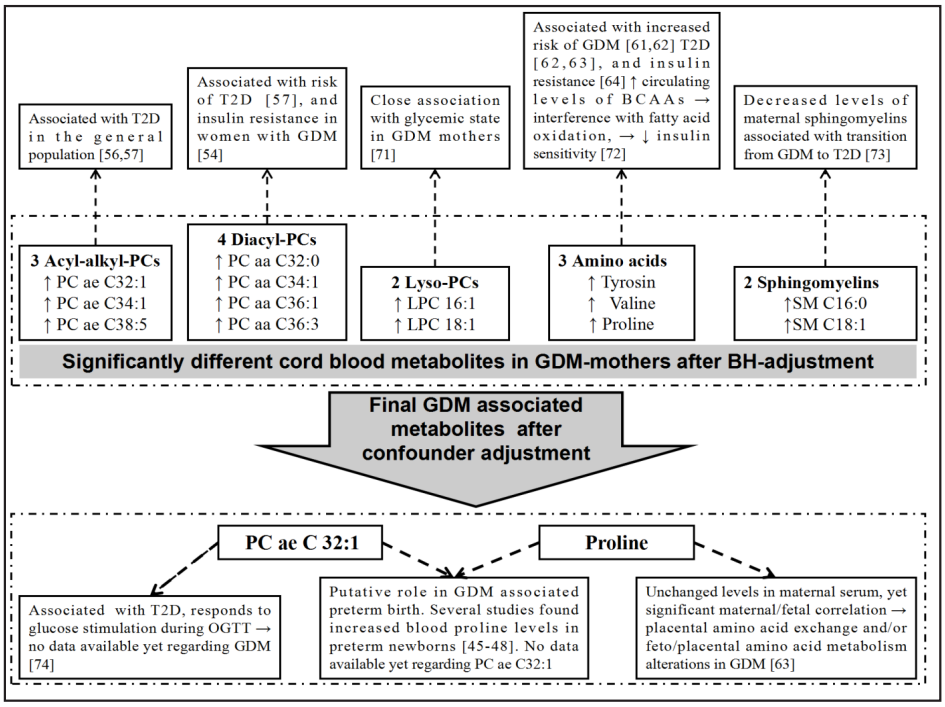

Fig. 3. Pathway of relevant metabolites for GDM. PC = phosphatidylcholine; ae = acyl-alkyl; SM = Sphingomyelins; OH = hydroxy; BCAAs = Branched-chain amino acids.

lifestyle-related environmental factors and medication with a possible influence on the metabolic profile, as well as the measurement of serum metabolites at multiple and/or earlier occasions. Metabolomic assessment of other easily accessible biological fluids, such as urine and feces might be of importance. Furthermore, the role of the preconceptual paternal metabolome in gestational disease such as GDM should be addressed by future studies. Lastly, GDM studies with a long-term follow up should be conducted, to evaluate lasting effects of early life metabolic alterations [67]. With a well-designed prospective study based on findings from this and other studies, a comprehensive understanding of the variation of metabolites in the development of GDM and possibly its consequences could be achieved.

Conclusion

This study aimed at finding characteristic metabolites in a mother-child cohort of well-controlled GDM and healthy pregnancies. There were no significant differences in the maternal metabolome between GDM and non-GDM mothers. Interestingly fetal cord blood phosphatidylcholine acyl-alkyl C 32:1 and proline were associated with GDM independent of established GDM risk factors. This finding of an independent association adds to the growing evidence in literature demonstrating a fetal impact on maternal gestational metabolic disease and warrants further research. Figure 3 gives a summary of the significantly different metabolites found in the current study and their association with GDM or T2D according to published literature.

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Disclosure Statement

No conflict of interests exists.

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