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DISSERTATION

Maternal and fetal metabolomic signatures in regard to birth outcome and gestational disease

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Summary

Abstract (English)

Background: The developmental origins of health and disease hypothesis states that an impaired intrauterine environment may induce long lasting changes in the offspring, affecting both, immediate birth outcomes and later life disease susceptibility. Impaired birth outcomes, such as low birth weight or preterm birth and also gestational diseases, such as gestational diabetes mellitus, have been associated with an increased risk for adult non communicable disease in the offspring. However, our understanding of the intrauterine environment and how it affects the phenotype of the offspring is still limited. Metabolomics, a robust and efficient method to analyze a large number of small molecules, could give new insight into the complex relationship between intrauterine conditions, maternal disease interactions and birth outcomes. The underlying aim of the study was to characterize the maternal and fetal metabolome at time of birth and to search for associations with birth outcomes (birth weight, preterm birth) and maternal gestational disease (gestational diabetes mellitus).

Methods: At the time of birth, serum samples from mothers and newborns (cord blood) were collected and screened for 163 metabolites utilizing tandem mass spectrometry in a mother/child pairs cohort. According to available data, subcohorts were created to analyze associations between the maternal/fetal metabolome and preterm birth, birth weight, and gestational diabetes mellitus. False discovery rate was adjusted by using the Benjamini-Hochberg procedure. To demonstrate independent association multivariable regression models were calculated adjusted for relevant confounding factors.

Results: Preterm birth was independently linked to higher diacyl-phosphatidylcholine 38:6 concentrations in maternal serum. Fetal lyso-phosphatidylcholines 14:0, 16:1, and 18:1 showed a strong and independent positive association with birth weight, No significant associations were observed between the maternal metabolome and birth weight. Gestational diabetes mellitus was independently associated with fetal cord blood concentrations of acyl-alkyl-phosphatidylcholine 32:1 and proline. There were no significant associations between maternal gestational diabetes mellitus and the maternal metabolome.

Conclusions: Specific birth outcomes and gestational diabetes were shown to be associated with maternal and fetal metabolic alterations. Results of the current studies showed a higher number of associations between birth outcomes/gestational disease and fetal metabolites. Thus, future studies with similar aims, should focus more on the so far neglected fetal metabolome.

Abstrakt (Deutsch)

Hintergrund: Die Hypothese der "developmental origins of health and disease“ beschreibt, dass ungünstige Einflüsse und Umweltbedingen während kritischer Phasen der intrauterinen Entwicklung zu langanhaltenden und irreversiblen Veränderungen bei den Nachkommen führen können. Die auf diese Weise induzierten Anpassungen in Organstruktur und –funktion beeinflussen sowohl neonatale anthropometrische Parameter als auch das spätere Krankheitsrisiko im Erwachsenenalter. Allerdings sind die komplexen zugrunde liegenden biologischen Vorgänge und molekularen Mechanismen bisher nur ansatzweise verstanden. Die Anwendung moderner Methoden wie der Massenspektrometrie erlaubt die Identifizierung und Quantifizierung einer großen Anzahl von Biomolekülen. So könnten neue Einblicke in die Wechselwirkungen zwischen intrauterinen Bedingungen, maternalen Krankheitheiten und neonatalem Outcome gewonnen werden. Ziel dieser Arbeit war es, maternale und fetale Metaboliten zum Zeitpunkt der Geburt zu bestimmen und nach Zusammenhängen mit den klinischen Parametern Geburtsgewicht und Frühgeburtlichkeit sowie Gestationsdiabetes zu suchen.

Methoden: Mittels Massenspektrometrie wurden mütterliche Serumproben vom Zeitpunkt der Geburt und Nabelschnurblut analysiert und 163 Metaboliten quantifiziert. Um Assoziationen zwischen maternalem und fetalem Metabolom und klinischen Parametern zu finden, wurden entsprechend der vorhandenen Daten Untergruppen gebildet und verschiedene multiple Regressionsmodelle gerechnet. Eine Kontrolle für falsch positive Ergebnisse wurde mit dem Benjamini-Hochberg Verfahren durchgeführt.

Ergebnisse: Frühgeburtlichkeit war unabhängig mit einer höheren Konzentration von Diacyl-phosphatidylcholin 38:6 im maternalen Serum assoziiert, während das Geburtsgewicht eine unabhängig positive Assoziation mit fetalem Lyso-phosphatidylcholin 14:0, 16:1, and 18:1 zeigte. Zwischen Geburtsgewicht bzw. Gestationsdiabetes und maternalen Metaboliten wurden keine signifikanten Zusammenhänge gefunden, jedoch war Gestationsdiabetes unabhängig mit fetalem Acyl-alkyl-phosphatidylcholin 32:1 und Prolin assoziiert.

Schlussfolgerungen: Die Ergebnisse dieser Studie zeigen, dass Parameter des neonatalen Outcomes bzw. Gestationsdiabetes mit Veränderungen des fetalen und maternalen Metaboloms assoziiert sind. Interessanterweise wurden für fetale Metabolite besonders häufig Zusammenhänge gefunden. In weiteren Studien sollte daher besonders das bis dato schlecht charakterisierte fetale Metabolom im Fokus der Untersuchungen stehen.

1. Introduction

It is by now widely accepted, that the early life environment is not just an important factor affecting immediate birth outcomes, but is also associated with disease susceptibility in later life. First observations in this regard originated from epidemiological studies that linked an impaired intrauterine environment - measured by a reduced weight at time of birth - with an increased risk for adult chronic, non-communicable disease [1,2]. These first observations were replicated in a large number of independent studies and lead to the hypothesis of the developmental origins of health and disease (DOHaD) [2-4]. Next to a reduced birth weight, newer evidence suggests that preterm birth (PTB) is similarly associated with an increased risk for later life non-communicable diseases, including metabolic and cardiovascular diseases [5,6].

Maternal pregnancy related diseases are known to impact on birth outcomes and also have been associated with long term risks for the offspring [7]. Gestational diabetes mellitus (GDM) is an important disease in this regard. It is defined as any degree of glucose intolerance with onset or first recognition during pregnancy. GDM is one of the most common complications of pregnancy and its prevalence is constantly rising [9]. If uncontrolled, GDM results in overt hyperglycemia, which may significantly increase perinatal morbidity and mortality [10-14]. Potential long-term consequences for the health of mother [7,15] and child [7] may be an impaired glucose tolerance, obesity, and metabolic disorders. Traditionally, GDM is consider as a result of environmental and maternal genotype predisposition [16,17]. Previous studies [18,19] also reported fetal might affect maternal physiology, even a risk of GDM [20]. The pathogenesis of GDM is not well understood so far.

Metabolomics is a fairly new ‘omics’ technology, which helps to characterize physiological conditions, systematically monitor environmental factors, genetic regulations, and enzymatic activity states [21,22]. Metabolomics may also serve to give new insight into the complex relationship between intrauterine conditions, maternal-fetal interactions, gestational diseases and birth outcomes. Furthermore, metabolomics may help in identifying relevant predictive biomarkers for impaired birth outcomes or gestational disease, such as PTB, birth weight and GDM. In this study a targeted metabolomics approach was performed, measuring 163 metabolites in maternal and fetal serum samples. Resulting metabolomics data were then used to find associations between certain metabolites and birth outcomes (PTB, birth weight) or

maternal gestational disease (GDM).

2. Methodology

2.1 Clinic data collection

The data compiled in this thesis stems from three studies performed with a subcohort (678 mother child pairs) of the Berlin birth cohort (BBC [23,24]) in which metabolomics was performed. Study 1 investigated associations between maternal serum metabolites and PTB. 550 mother child pairs were included in this study. In the following two studies the focus was shifted from solely analyzing maternal metabolites to also investigate the fetal metabolome. Study 2 investigated associations between maternal and fetal metabolites and birth weight. 226 mother child pairs were included in this study. Study 3 analyzed associations between maternal and fetal metabolites and GDM. 412 mother child pairs were included in this study. assessed screening for GDM was performed according to the practice guideline of the German Diabetes Association (DDG) and the German Association for Gynecology and Obstetrics (DGGG) [25]. In the analyzed cohort, 31 of the 412 pregnant women were diagnosed with GDM.

Maternal basic and clinic data (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.) and newborn biometric data (birth weight, birth length, ponderal index (birth length (m) / the cube root of weight (kg)), head circumference, child sex, and Apgar score 5 & 10 minutes.) were collected.

2.2 Sample collection

Before delivery, midwives collected maternal blood from the cubital vein, and collected umbilical cord immediately after delivery in the delivery room or on the ward. After collection, the blood samples were centrifuged at 2750 g immediately. All obtained serum was stored at -80 °C until metabolites measurement.

Out of the 678 mother child pairs that entered the study, metabolomics was performed in 550 maternal and 412 fetal cord blood samples. In study 1, which focused on screening for PTB

biomarkers, metabolic data of 550 mothers was investigated. In study 2 and 3, both maternal and fetal metabolites were analyzed. Study 2 investigated associations between maternal and fetal metabolites and birth weight, and also studied interactions between the maternal and fetal metabolome, resulting in the data analysis of 226 mother/child pairs. Study 3 investigated associations between maternal and fetal metabolites and GDM, yet due to the lack of data in literature in regard to fetal cord blood metabolites and GDM, the main focus was set on the available metabolomic data of 412 fetal cord blood samples.

2.3 Targeted metabolomics in blood serum

Metabolomic measurements were performed in the Metabolomic Platform of the Genome Analysis Center, Helmholtz Zentrum München. The quantification of 163 metabolites were assayed by FIA-ESI-MS/MS and the Absolute*IDQ*™ p150 Kit (BIOCRATES Life Sciences AG, Innsbruck, Austria) out of 10 µL serum. All metabolite concentrations were reported in µM. In total, the 163 metabolites including free carnitine, 40 acylcarnitines, 14 amino acids (13 proteinogenic + ornithine), hexoses (sum of hexoses – about 90-95 % glucose), 92 glycerophospholipids (15 lyso-phosphatidylcholines (lysoPC) and 77 phosphatidylcholines (PC)), and 15 sphingolipids. The method of Absolute*IDQ*™ p150 Kit has been proven to be in conformance with the EMEA-Guideline "Guideline on bioanalytical method validation (July 21st 2011") [26], which implies proof of reproducibility within a given error range. A detailed description of the sample preparation, assay procedures and nomenclature have been published previously [27,28]. For more details of the sample handling, mass spectrometric analyses, data evaluation, and quality assessment see publication 1, 2, and 3.

2.4 Statistical analysis

Statistical analysis was performed by SPSS version 22.0. Quantitative data results of were expressed as the arithmetic mean ± standard deviation (SD). Continuous variable data between two groups were compared by unpaired t-test. Qualitative data were analyzed by Pearson's chi-square test. The linear correlation between quantitative data were analyzed by bivariate correlation analysis. In order to reduce false discovery rate (FDR), *P*-values were adjusted with Benjamini-Hochberg (BH) procedure. Significant difference of BH procedure is defined as $P_m \leq m \times q/M$ [29,30]. M equals the total number of tested metabolites (M=163), q equals the FDR (the

FDR is 0.05 in the current study), *P*m equals the individual *P*-value's rank, and m equals the individual rank of tested metabolite. Significant difference was considered as a *P*-value less than 0.05. A flow diagram of the three studies analysis strategy is given in Figure 1.

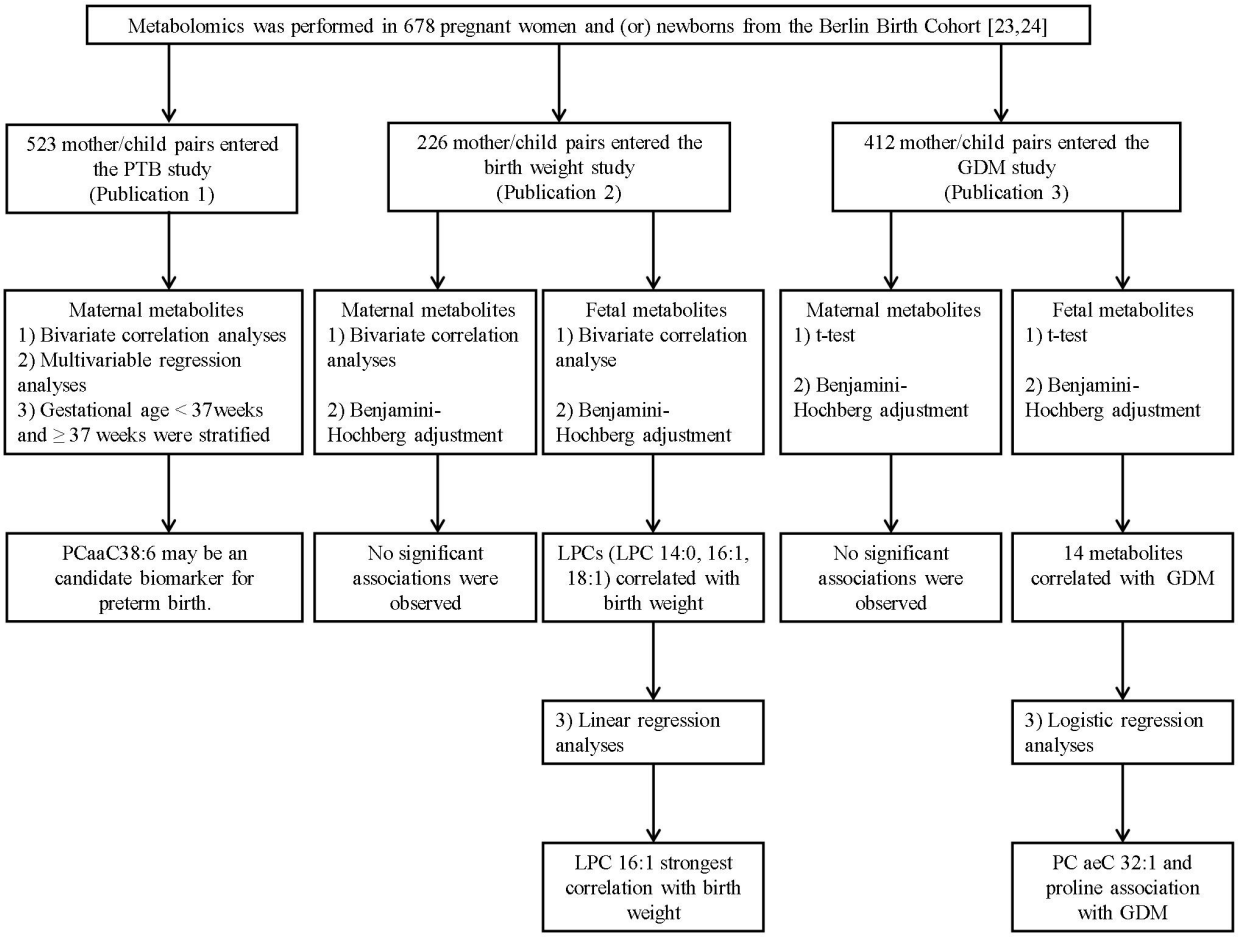

Figure 1. Flow diagram of the three studies analysis strategy. PTB = preterm birth; GDM = gestational diabetes. LPC = lysophosphatidylcholine; PC = phosphatidylcholine; ae = acyl-alkyl. Note. The number of participants in the three studies were different, because we used different analytic methods and different clinical data sets depending on the specific topic of the studies. Fetal metabolomics was only performed in a subset of the 678 mothers with available metabolomic data.

3. Results

Publication 1: "Maternal PC aa C38:6 is associated with preterm birth - a risk factor for early and late adverse outcome of the offspring"

Maternal serum metabolites and PTB

For more details see publication 1 (page 25)

Summary

Bivariate correlation analysis and multivariable regression analysis models adjusted for maternal BMI before pregnancy, smoking during pregnancy, systolic blood pressure at the third trimester, maternal BMI at the third trimester showed that PC aa C38:6 was inversely correlated with gestational age. This result was further checked by unpaired t-test: group 1, gestational age < 37 weeks (PTB group); group 2, gestational age ≧ 37 weeks. The PTB group showed significantly higher levels of PC aa C38:6 when compared with group 2 (100.09 ± 4.22 μM vs. 89.66 ± 1.17 μM, $P = 0.023$).

Publication 2: "Cord blood lysophosphatidylcholine 16: 1 is positively associated with birth weight"

Maternal serum metabolites and child birth weight

For more details see publication 2 (page 33)

Summary

Employing bivariate correlation analysis followed by BH adjustment, there was no maternal serum metabolite associated with child birth weight ($P_m > m \times q/M$).

Fetal serum metabolites and child birth weight

Summary

Following bivariate correlation analysis and BH procedure, multiple linear regression analysis (considering known factors associated birth weight, such as gestational age [31], child sex [32], maternal age [33], maternal BMI before pregnancy [34], maternal smoking during pregnancy [35] as confounders), showed that fetal serum LPC 14:0, 16:1, and 18:1 were significantly associated with birth weight. Stepwise models of multiple linear regression were conducted to identify which of the of the three LPCs (LPC 14:0, 16:1 and 18:1) has the strongest impact on birth weight. These analyses demonstrated that fetal serum LPC 16:1 showed the strongest correlation with birth weight in the stepwise regression models.

Publication 3: "Fetal serum metabolites are independently associated with gestational diabetes mellitus"

Maternal serum metabolites and gestational diabetes

For more details see publication 3 (page 44)

Summary

Unpaired t-test comparisons of mothers with GDM and mothers without GDM showed no significant differences in maternal serum metabolite concentrations after BH adjustment ($P_m >$ m×q/M).

Fetal serum metabolites and gestational diabetes

Summary

Following BH adjusted t-tests comparing fetal cord blood metabolites from offspring of diabetic to offspring of non-diabetic mothers, logistic regression models (considering known factors associated with GDM, such as maternal age, ethnicity, family history of diabetes, pre-pregnancy BMI, and smoking during pregnancy [36,37] as confounders), showed independent associations between fetal serum PC ae C 32:1 or the amino acid proline with GDM.

As it was shown that gestational age may impact on the fetal and maternal metabolome [38], an additional model was calculated including gestational age as confounder. Also in this model fetal serum PC ae C 32:1 and the amino acid proline still demonstrated an independent association with GDM.

4. Discussion

The study aimed at investigating associations between maternal and fetal serum metabolites and birth outcomes such as gestational age at birth and child birth weight. Another aim was to find characteristic metabolites of well-controlled GDM. Maternal serum concentrations of PC aa C38:6 were associated with PTB. There were no associations between maternal serum metabolites and birth weight or GDM. However, fetal serum LPCs 14:0, 16:1, and 18:1 were significantly and positively correlated with birth weight. Furthermore, fetal serum PC ae C 32:1 and the amino acid proline showed an independent association with GDM.

LPCs and PCs are clusters of glycerophospholipids which make up the main structural element of most eukaryotic membranes [39,40]. PCs are secreted and biosynthesised in the liver whereas [40] LPCs are derived from PCs hydrolysis by the enzymatic action of phospholipase A2 [41]. So far the role of PCs and LPCs during intrauterine life is very incompletely understood. In the current studies, variations of maternal or fetal glycerophospholipids were independently associated with PTB, birth weight, and maternal GDM.

Publication 1: "Maternal PC aa C38:6 is associated with preterm birth - a risk factor for early and late adverse outcome of the offspring"

Two previous studies identified characteristic metabolites for PTB in amniotic fluid samples, including carbohydrates, amino acids [42] and many metabolites reflecting liver metabolism [43] to be associated with PTB. Different from the former two studies, the present study investigated maternal serum metabolites to investigate associations between maternal serum metabolites and PTB. Compared with normal mothers with a normal course of pregnancy, mothers with PTB demonstrated significantly higher levels of PC aa C38:6.

Several factors capable of causing redox imbalances, such as maternal malnutrition, smoking, obesity, intra-amniotic infection and inflammation, have been associated with PTB [44-46]. Oxidative stress induced damage plays an important role in the premature rupture of membranes [47-49]. A possible link between increased oxidative stress, e.g. due to smoking [50] and PTB might be alterations in metabolite levels. Xu *et al.* [51] demonstrated in a large cohort study that current smokers had higher levels of unsaturated diacyl-PCs when compared with former smokers and never smokers. In the present study unsaturated diacyl-PCs (PCaaC36:4, PCaaC38:4, PCaaC38:5, PCaaC38:6, PCaaC40:4, PCaaC40:5, PCaaC40:6, PCaaC42:4), especially PCaaC38:6, were negatively associated with gestational age even after adjusting for maternal smoking status during pregnancy. Compared to the term birth group, the PTB group showed significantly higher levels of PC aa C38:6. Higher levels of the unsaturated diacyl-PCs may indicate higher levels of oxidative stress, which may be involved in the pathogenesis of PTB. The measurement of unsaturated diacyl-PCs might serve as a biomarker for PTB, yet appropriately designed future studies are still needed to confirm this.

Publication 2: "Cord blood lysophosphatidylcholine 16: 1 is positively associated with birth weight"

In agreement with a recent study [52], the present study demonstrated that fetal LPCs were independently associated birth weight. In the large birth cohort study [52], several cord blood LPCs, including LPC 14.0, 16:1, and 18:1 were independently positively correlated with birth weight. The current study confirmed these results using a strict statistical approach the including BH procedure and stepwise multiple linear regression models adjusted for relevant confounding factors.

Increasing evidence in epidemiological studies [53-57] and animal studies [58] shows a relationship between LPCs and overweight/obesity. However, study results in this regard are conflicting, showing both positive and negative associations between obesity and LPCs. An explanation for the contradictive results of these studies [53-58] might be due to the number and specific types of analyzed LPCs, as saturated and unsaturated LPCs might exert different biological functions [59-61]. It was shown in human or animal studies that obesity was associated with lower concentrations of the unsaturated LPC 18:1 [55-58] and higher levels of saturated LPCs (LPC 14:0, 18:0) [57] compared to lean controls. Another study performed in children demonstrated that rapid growth in infancy and childhood obesity was positively related to LPC 14:0 [54]. It is also known that subcutaneous adipose tissue mass is lower in growth-restricted (GR) infants than appropriate-for-gestational-age (AGA) infants [62], and extremely preterm birth with significantly lighter weight [63]. In the current study, lower birth weight newborns demonstrated significantly lower levels of serum LPCs 14:0, 16:1 and 18:1, Based on these observations, this may indicate that during early life development LPCs might be involved in cell division, adipose tissue growth and remodeling, potentially beneficially affecting fetal intrauterine growth.

LPCs were also shown to be connected to insulin signaling. LPCs were reported to stimulate adipocyte glucose uptake and improve glucose homeostasis [64] and enhance glucose-dependent insulin secretion [65]. Furthermore, a correlation between lower levels of LPC and insulin resistance was reported in women with GDM [66]. In regard to the interpretation of the current study these findings are interesting, as there is evidence that fetal insulin resistance may be related to decreased birth weight [67,68]. In the current study, lower levels of LPCs (especially

LPC 16:1) were associated with lower birth weight. Hypothetically, low levels of cord blood LPCs might be associated with insulin resistance and decreased insulin secretion, and thus influence fetal growth.

Publication 3: "Fetal serum metabolites are independently associated with gestational diabetes mellitus"

Until now there are only a few studies that investigated the fetal metabolome in regard to maternal GDM [69]. A previous study [66], analyzing maternal plasma metabolites, demonstrated that lower levels of diacyl-PCs, LPCs and arachidonic acid were associated with insulin resistance in women with GDM. The current study not solely analyzed potential relationships between maternal metabolites and GDM, but also investigated associations between fetal cord blood metabolites and GDM. Maternal serum metabolites were not associated with GDM in the current study. However, fetal serum PC ae C 32:1 and the amino acid proline were independently associated with GDM, even after BH procedure and adjustment for established risk factors of GDM.

There is increasing evidence [70-73] reporting that changes of diacyl-PC and acyl-alkyl-PC are associated with type 2 diabetes in the general population. Elevated concentrations of branched-chain amino acids were also associated with an increased risk of GDM [74,75] T2D [74,76], and insulin resistance [75]. To the best of our knowledge, this is the first large scale study that identified the fetal serum acyl-alkyl-PC 32:1 and the amino acid proline to be independently associated with maternal GDM. The pathogenesis of GDM is incompletely understood so far. It is believed that GDM results from a complex interaction of multiple environmental stimuli and maternal factors, with the placenta playing a key role [16,17]. Studies [18,19] propose that the fetal genetic setup may also impact on maternal physiology and pathophysiology during pregnancy, including maternal metabolism. The mechanisms that underlie a fetal influence on maternal metabolism during gestation are still very incompletely understood. The fetal genome might affect secretion patterns of placental hormones resulting in variations of placental function [18,19], thus potentially impacting on maternal glucose status. Metabolomics is a predictive approach that summarizes the impact of genetic regulation, physiological conditions, and environmental factors on the metabolome [21,22]. Altered levels of

fetal cord blood metabolites may be indicative of fetal genetic variants that are associated with maternal GDM. However, this hypothesis has to be confirmed by future prospective studies, integrating maternal and fetal genetic data and information of lifestyle-related environmental factors and associate these with characteristic metabolomic alterations.

Conclusion

The performed studies identified maternal and fetal serum metabolites that were associated with PTB, birth weight or GDM. Especially the fetal metabolome seems of interest, as more and stronger associations were found analyzing fetal metabolites than by the analysis of maternal metabolites. Given that there still is a lack of studies focusing on the fetal metabolome, the variety of associations between fetal metabolites and birth outcomes/gestational disease show that further research is warranted. These findings add to the growing evidence in literature, demonstrating a maternal impact on fetal intrauterine development, yet also highlight a potential fetal impact on maternal gestational disease. Given the purely associative character, the current study result needs confirmation by future well designed clinical and animal studies.

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Affidavit

I, Yongping Lu, certify under penalty of perjury by my own signature that I have submitted the thesis on the topic "*Maternal and fetal metabolomic signatures in regard to birth outcome and gestational disease*" I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The section on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) corresponds to the URM (s.o) and are answered by me. My contribution in the selected publication for this dissertation corresponds to those that are specified in the following joint declaration with the responsible person and supervisor.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Date..... Signature.....

Declaration of own contributions

YongPing Lu had the following share in the following publications:

* contributed equally

Publication 1:

*Li J, *Lu YP, Reichetzeder C, Kalk P, Kleuser B, Adamski J, Hocher B. Maternal PC aa C38:6 is associated with preterm birth - a risk factor for early and late adverse outcome of the offspring. Kidney Blood Press Res. 2016;41(3):250-7. (Impact Factor: 3.104)

Contribution in detail:

Contributed to the idea and conception of the article, performed alone after training all biochemical analysis related to metabolomics using mass tandem spectroscopy, this is the key biochemical technology of the study. Also analyzed all data, performed statistics what is very complex in metabolomic studies and finally created the tables and figures of the manuscript. I also drafted the first version of the manuscript that was thereafter circulated for corrections among the coauthors.

Publication 2:

*Lu YP, *Reichetzeder C, Prehn C, Yin LH, Yun C, Zeng SF, Chu C, Adamski J, Hocher B. Cord blood lysophosphatidylcholine 16:1 is positively associated with birth weight. Cell Physiol Biochem. 2018;45:614-624. (Impact Factor: 5.104)

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Contributed to the idea and conception of the article, performed alone after training all biochemical analysis related to metabolomics using mass tandem spectroscopy, this is the key biochemical technology of the study. Also analyzed all data, performed statistics what is very complex in metabolomic studies and finally created the tables and figures of the manuscript. I also drafted the first version of the manuscript that was thereafter circulated for corrections among the coauthors.

Publication 3:

*Lu YP, *Reichetzeder C, Prehn C, Websky KV, Slowinskib T, Chen YP, Yin LH, Kleuser B, Yang XS, Adamski J, Hocher B. Fetal serum metabolites are independently associated with gestational diabetes mellitus. Cell Physiol Biochem. 2018;45:625-638. (Impact Factor: 5.104)

Contribution in detail:

Contributed to the idea and conception of the article, performed alone after training all biochemical analysis related to metabolomics using mass tandem spectroscopy, this is the key biochemical technology of the study. Also analyzed all data, performed statistics what is very complex in metabolomic studies and finally created the tables and figures of the manuscript. I also drafted the first version of the manuscript that was thereafter circulated for corrections among the coauthors.

Signature, date and stamp of the supervising University teacher

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Signature of the doctoral candidate

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Original Paper

Maternal PCaaC38:6 is Associated With Preterm Birth – a Risk Factor for Early and Late Adverse Outcome of the Offspring

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Key Words
Metabolomics • PCaaC38:6 • Biomarker • Preterm birth

Abstract
Background/Aims: Preterm birth (PTB) and low birth weight (LBW) significantly influence mortality and morbidity of the offspring in early life and also have long-term consequences in later life. A better understanding of the molecular mechanisms of preterm birth could provide new insights regarding putative preventive strategies. Metabolomics provides a powerful analytic tool to readout complex interactions between genetics, environment and health and may serve to identify relevant biomarkers. In this study, the association between 163 targeted maternal blood metabolites and gestational age was investigated in order to find candidate biomarkers for PTB. ***Methods:*** Five hundred twenty-three women were included into this observational study. Maternal blood was obtained before delivery. The concentration of 163 maternal serum metabolites was measured by flow injection tandem mass spectrometry. To find putative biomarkers for preterm birth, a three-step analysis was designed: bivariate correlation analysis followed by multivariable regression analysis and a comparison of mean values among gestational age groups. ***Results:*** Bivariate correlation analysis showed that 2 acylcarnitines (C16:2, C2), 1 amino acids (xLeu), 8 diacyl-PCs (PCaaC36:4, PCaaC38:4, PCaaC38:5, PCaaC38:6, PCaaC40:4, PCaaC40:5, PCaaC40:6, PCaaC42:4), and 1 Acylalkyl-PCs (PCaeC40:5) were inversely correlated with gestational age. Multivariable regression analysis confounded for PTB history, maternal body mass index (BMI) before pregnancy, systolic blood

Jian Li and Yong Ping Lu contributed equally to this study and therefore share first authorship.

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pressure at the third trimester, and maternal body weight at the third trimester, showed that the diacyl-PC PCaaC38:6 was the only metabolite inversely correlated with gestational age. ***Conclusions:*** Maternal blood concentrations of PCaaC38:6 are independently associated with gestational age.

Introduction

Preterm birth (PTB), defined as birth before 37th week of gestation, affects 5 to 18% of pregnancies worldwide. Evidence suggests that PTB not only significantly influences mortality and morbidity of the offspring in early life [1-7] but also causes serious long-term consequences in later life [8-11]. Systematic reviews and meta-analyses including large populations and different separate cohorts demonstrated that PTB increased the risk of metabolic syndrome at later childhood and adulthood [8-10]. Sensitive, effective, and non-invasive new biomarkers to screen and diagnose PTB before birth could lead to treatment options that prevent poor birth outcomes associated with PTB. Pregnancy is a very complex process, influenced by a plethora of physiological and pathophysiological factors. It is known that environmental factors like maternal infections, nutrition, and stressful events can be associated with changes in the serum metabolite profile. Metabolomics provides a powerful analytic tool to get insights into the complex interaction of genetics, environment and health and may serve to identify relevant predictive biomarkers for PTB. Previous work from *Romero et al.* [12] and *Menon et al.* [13] showed that the metabolomic profile of amniotic fluid is a good biomarker to assess the risk of preterm delivery. However, amniotic fluid sample collection is an invasive procedure with potential risk for adverse outcomes for both mother and child [14,15]. Blood plasma and (or) urine can be collected much easier and therefore should be targeted in the search of potential biomarkers. One recent study demonstrated that the maternal urinary metabolic profile in early pregnancy can be helpful to identify PTB [16]. So far, there is no publication about the metabolomic profile of maternal plasma and PTB. In this study, a targeted metabolomics approach was performed, measuring 163 metabolites which were analyzed for associations with gestational age. The detection of new biomarkers predictive for PTB could help to better understand the underlying pathomechanisms of PTB and to create new preventive strategies for PTB.

Materials and Methods

Clinic data collection

This observational study was approved by the local Ethics Committee. A total of 550 pregnant women who delivered their babies at the Charité obstetrics department in Berlin, Germany between January 2007 and December 2008 were invited to participate. As 27 mothers were excluded from the study because they delivered twins, 523 women entered the study. The majority of the mothers (n = 471) were of caucasian ethnicity, 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, body weight before pregnancy, gravidity, parity, diabetes mellitus and hypertension during pregnancy, smoking status before and during pregnancy, systolic and diastolic blood pressure (BP) measurements recorded during pregnancy and the mode of delivery. Biometric data of the newborn were collected during the routine postnatal examination: birth weight, birth length, head circumference, child sex, and Apgar score 5 minutes postnatally and Apgar score 10 minutes postnatally. Gestational age at delivery was based on last menstrual period, anamnestically assessed during the first pregnancy examination.

Sample collection, blood metabolomics compounds assay

Midwives collected maternal blood from a cubital vein in the delivery room or on the ward prior to

birth, before the usage of oxytocin, or analgesics. Blood was centrifuged at 2750 g immediately after its taking and the obtained serum was stored at -80 °C until measurements were performed.

163 targeted small metabolites were quantified simultaneously in 10 µL of serum using the Absolute IDQTM-kit p150 (Biocrates Life Sciences AG, Innsbruck, Austria). The assay procedures were the same as previously described [17]. Concentrations of these targeted metabolites were recorded in µM. These metabolites included 14 amino acids, 1 sugar, 1 carnitine, 26 acylcarnitines, 14 hydroxy and dicarboxy-acylcarnitines, 10 sphingomyelins, 5 hydroxy-sphingomyelins, 38 diacyl-phosphatidylcholines (diacyl-PCs), 39 acyl-alkyl-phosphatidylcholines (Acylalkyl-PCs) and 15 lysophosphatidylcholines.

Table 1. Detailed Descriptive Data of the Mother/Child Pairs (n = 523)

Variable	Mean±SE/%
Maternal age, y	30.78±0.26
Maternal height, cm	166.62±0.29
Maternal weight before pregnancy, kg	62.99±0.59
Maternal BMI before pregnancy, kg/m^2	22.66±0.20
Primigra/pluripara, %	37.37/62.63
Smoking before/during pregnancy, %	43.07/16.18
Hypertension before/during pregnancy, %	4.40/10.33
Diabetes mellitus before/during pregnancy, %	0.96/8.60
Mean weight 1st half of pregnancy, kg	66.12±0.70
Mean weight 2nd half of pregnancy, kg	69.11±0.63
Mean weight 3rd half of pregnancy, kg	76.19±0.62
Mean systolic BP 1st half of pregnancy, mm Hg	113.20±0.63
Mean systolic BP 2nd half of pregnancy, mm Hg	112.58±0.53
Mean systolic BP 3rd half of pregnancy, mm Hg	114.23±0.47
Mean diastolic BP 1st half of pregnancy, mm Hg	68.42±0.45
Mean diastolic BP 2nd half of pregnancy, mm Hg	67.40±0.35
Mean diastolic BP 3rd half of pregnancy, mm Hg	69.26±0.33
Gestational age at delivery, weeks + days	38+3
Child sex, male/female, %	52.18/47.82
Child birth weight, g	3257.53±28.21
Child birth length, cm	50.03±0.18
Head circumference, cm	34.53±0.08
Apgar score at 5 min	9.21±0.05
Apgar score at 10 min	9.48±0.04

Data are given as mean ± SE or %

Statistical analysis

Data were analyzed with SPSS version 17.0. Results of quantitative data were expressed as arithmetic mean ± standard error (SE). Bivariate Correlation Analysis was applied to detect correlations of metabolites and gestational age. Additionally, certain factors (PTB history, maternal BMI before pregnancy, systolic blood pressure at the third trimester, maternal body weight at the third trimester) were used as confounders to calculate and adjust putative predictive metabolites in multivariable linear regression models. Unpaired t-test was used for comparison of continuous variables between two groups. 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, biometric data of the newborns like birth weight, birth length, child sex, and Apgar score (for more details, see Table 1). The distribution of gestational age is given in Figure 1.

Bivariate correlation analyses of maternal serum metabolites and gestational age

Bivariate correlation analyses showed that 2 acylcarnitines (C16:2, C2), 1 amino acid (xLeu), 8 diacyl-PCs (PCaaC36:4, PCaaC38:4, PCaaC38:5, PCaaC38:6, PCaaC40:4, PCaaC40:5, PCaaC40:6, PCaaC42:4), and 1 acylalkylphosphatidylcholine (PCaeC40:5) were significantly negatively correlated with gestational age (for more details, see table 2).

Multivariable regression analyses of maternal blood metabolites and gestational age

Significant results obtained by bivariate correlation analysis were subsequently analy-

zed by multivariable regression analysis considering PTB history, maternal BMI before pregnancy, systolic blood pressure at the third trimester, maternal BMI at the third trimester in model B. Analysis showed that PCaaC38:6 was the only metabolite significantly inversely correlated with gestational age (for more details, see table 3).

PCaaC38:6 serum concentrations in relation to gestational age

2 gestational age groups were stratified as follows: gestational age < 37weeks as group 1 (PTB group), ≧ 37 weeks as group 2. The PTB group displayed significantly higher concentrations of PCaaC38:6 in comparison to group 2 (100.09 ± 4.22 μM vs. 89.66 ± 1.17μM, $P = 0.023$);

4 gestational age groups were stratified as follows: gestational age < 35 weeks as group 1 (severe PTB group), 35~37 weeks as group 2 (moderate PTB group), 38~39 weeks as group 3, ≧ 40 weeks as group 4. PCaaC38:6 showed a trend to decrease with increasing gestational age (for more details, see figure 2).

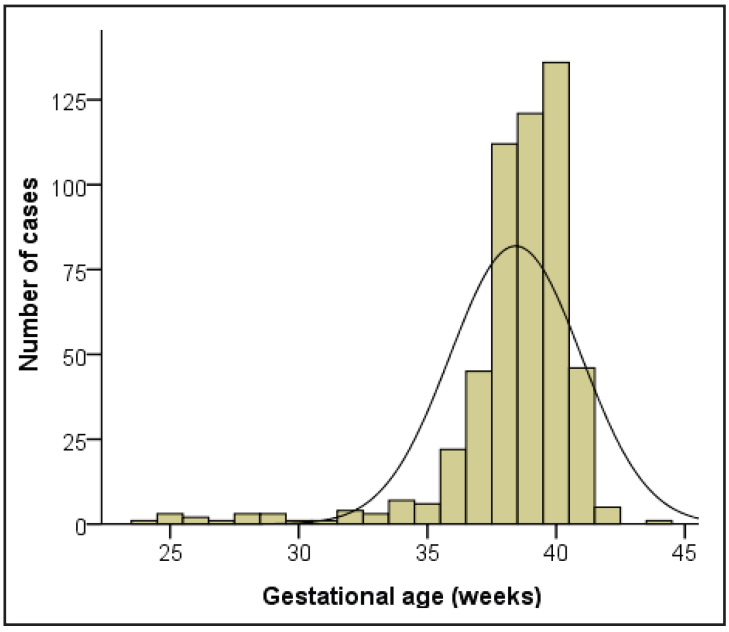

Fig. 1. Distribution of gestational age.

Table 2. Maternal serum metabolites related to gestational age in bivariate correlation analysis

	Variable	Pearson Correlation	*P* value
Acylcarnitines	C16:2	-0.163	1.189×10^{-4}
	C2	-0.122	0.004
Amino acids	xLeu	-0.091	0.033
Diacyl-PCs	PCaaC36:4	-0.104	0.014
	PCaaC38:4	-0.111	0.009
	PCaaC38:5	-0.088	0.040
	PCaaC38:6	-0.122	0.004
	PCaaC40:4	-0.089	0.037
	PCaaC40:5	-0.094	0.027
	PCaaC40:6	-0.120	0.005
	PCaaC42:4	-0.084	0.048
Acylalkyl-PCs	PCaeC40:5	-0.108	0.012

For each maternal serum metabolite, the bivariate correlation analysis was performed, with the maternal serum metabolite values being the dependent and each of the maternal gestational age being the independent variable. Only the 12 significant correlated metabolites are shown here. Diacyl-PCs : diacyl-phosphatidylcholines; Acylalkyl-PCs: acyl-alkyl-phosphatidylcholines

Discussion

In the current birth cohort study, the association of 163 maternal serum metabolites and gestational age before delivery was investigated. Diacyl phosphatidylcholine PCaaC38:6 was strongly and inversely correlated with gestational age. Concentration of PCaaC38:6 was significantly higher in serum of mothers with PTB than in the serum of mothers who gave birth between 37th and 40th or above 40th week of gestation. This indicates that unsaturated diacyl-PCs (PCaaC38:6) might be candidate biomarkers for PTB screening and monitoring.

In recent years, elaborate molecular biologic methods like genomics, transcriptomics, and proteomics have been used to study normal pregnancy and pregnancy complications. Yet, only two studies have reported the use of metabolomics in PTB. Romero et al. [12]

Table 3. Maternal serum metabolites related to gestational age in multivariable linear regression analysis

Variable	B	t	*P*	95.0% Confidence interval for B
PCaaC38:6	-0.04	-2.19	0.029	-0.072～-0.004
PTB history	-15.98	-4.14	4.06×10^{-5}	-23.56～-8.40
Maternal BMI before pregnancy	-0.51	-2.93	0.017	-0.93～-0.09
Systolic blood pressure at the third trimester	-0.27	-4.32	4.09×10^{-5}	-0.39～-0.14
Maternal body weight at the third trimester	0.31	3.98	7.83×10^{-5}	0.16～0.46

Considering PTB history, maternal BMI before pregnancy, systolic blood pressure at the third trimester, maternal body weight at the third trimester, the 4 metabolites shown in table 2 (P<0.01) being the independent variable and gestational age being the dependent variable. Only PCaaC38:6 was significant correlated with gestational age. Shown are the B, t, and P-values for the maternal gestational age. B: non-standardized regression coefficient.

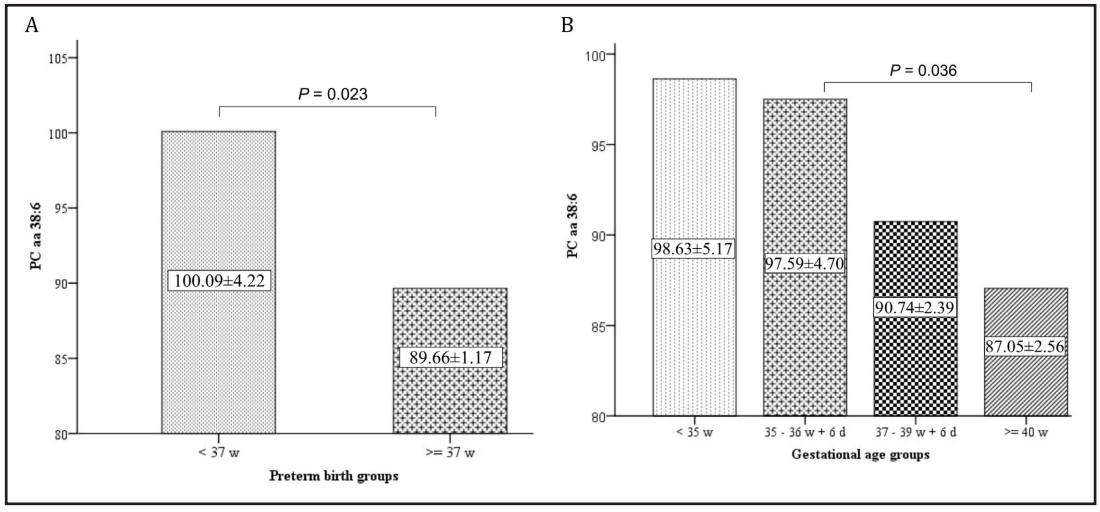

Fig. 2. Mean maternal PCaaC38:6 according to gestational age groups. A: 2 gestational age groups were stratified as follows: <37 weeks with 57 cases, ≧ 37 weeks with 466 cases. B: 4 gestational age groups were stratified as follows: <35 weeks with 29 cases, 35～37 weeks with 73 cases, 38～39 weeks with 233 cases, ≧ 40 weeks with 188 cases.

collected amniotic fluid from transabdominal amniocentesis at the time of diagnosis for preterm labor and showed that mothers without intraamniotic infection/inflammation who delivered preterm had a relative decrease in carbohydrates and amino acids. In contrast, mothers with intraamniotic infection/inflammation had a more substantial decrease in compounds of the carbohydrate cluster, and a relative increase in amino acids. Menon et al. [13] collected amniotic fluid from transvaginal amniocentesis samples taken prior to delivery during active labor in an African-American population and found that the global metabolite profiles differed significantly between normal and preterm birth. Many of the significantly altered metabolites in the PTB group reflected liver metabolism. Different to previous studies, the current study investigated maternal metabolites in serum obtained before delivery in a typical German cohort. Statistical analyses showed that maternal plasma phospholipids (six Diacyl-PCs and two Acylalkyl-PCs), especially PCaaC38:6, are relevant to PTB.

Glycerophospholipids make up the main structural lipids of cellular membranes, and phosphatidylcholine (PC) accounts for >50% of the glycerophospholipids in most eukaryotic membranes [18, 19]. PC is the main component of circulating lipoprotein classes in the human plasma [20]. In addition, PC is particularly essential for hepatic secretion of

triglyceride-rich very low density lipoprotein (VLDL) and high density lipoprotein (HDL) [19]. PC is essential for proper cell division and thus plays a very important role during fetal intrauterine growth. Furthermore, PC is the main source of choline which is essential for fetal brain and neurocognitive development [21,22]. Bernhard et al. [23] showed that in preterm infants, choline concentrations are lower in postnatal plasma than in cord plasma. La et al [24] conducted an S-plot for biomarkers of gestational age (PCs, PEs, and SMs) and showed that they had a weak, negative correlation with gestational age. Till now, there is no data available that links diacyl-PCs to gestational physiological or pathological conditions.

The most common phenotype of PTB is spontaneous PTB of unknown etiology. Risk factors of PTB include malnutrition, obesity, intra-amniotic infection (IAI) and inflammation, cigarette smoking, alcohol intake, drug use, antioxidant deficient diets, physiologic and psycho-social stressors, environmental pollutants, genotoxic agents, geographic location [25]. All of the PTB risk factors are capable of causing redox imbalances, leading to the production of superoxide, hydrogen peroxide, hydroxyl ions and nitric oxide that can damage collagen matrix and consume antioxidant defenses. These events can trigger uterine contractions (labor), leading to PTB and placing these infants at a higher risk of injury [25]. Recently, a large cohort study with 18,079 participants (KORA study) and seven years follow-up [26] showed that compared with former smokers and never smokers, current smokers had higher concentrations of unsaturated diacyl-PCs but lower concentrations of saturated diacyl-PC. It has been shown that unsaturated fatty acids are more vulnerable to lipid peroxidation [27-29]. Furthermore, polyunsaturated diacyl-PCs can promote the oxidation and fragmentation of γ-hydroxyalkenals [29]. Oxidative stress (OS) can occur early in pregnancy [30], induce pregnancy-related disorders like preeclampsia [31-33] and preterm premature rupture of membranes [34-36], and is considered a detrimental factor in preterm birth pathology [25]. Results of the current study showed that unsaturated diacyl-PCs (PCaaC36:4, PCaaC38:4, PCaaC38:5, PCaaC38:6, PCaaC40:4, PCaaC40:5, PCaaC40:6, PCaaC42:4), especially PCaaC38:6, were negatively correlated with gestational age. Higher unsaturated diacyl-PCs may be indicative of a higher level of OS. Future studies, including animal experiments and human epidemiological studies are needed to confirm the relationship between diacyl-PCs and OS.

One limitation of the study is that maternal serum metabolites were only measured prior to birth. Given that a predictive biomarker for PTB could be of great clinical significance, the current results should stimulate studies, which focus on measuring serum metabolites at multiple and/or earlier occasions in order to consider a potential gestational age dependent secretion of the metabolomic biomarkers. In particular the lipid biomarkers identified in the current study need confirmation in a second independent prospective study. Moreover, our work should stimulate preclinical work using specific tools to block and up-regulate the biological action of PCaaC38:6 to reveal causality and biological relevance between PCaaC38:6 and PTB.

Conclusion

Maternal blood PCaaC38:6 was clearly correlated with gestational age. PCaaC38:6 may be a candidate biomarker for PTB.

Disclosure Statement

The authors of this manuscript state that they do not have any conflict of interests and nothing to disclose.

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Original Paper

Cord Blood Lysophosphatidylcholine 16:1 is Positively Associated with Birth Weight

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Key Words
Metabolomics • Lysophosphatidylcholine • Birth Weight • DOHaD • Hypertension • Type 2 Diabetes

Abstract
Background/Aims: Impaired birth outcomes, like low birth weight, have consistently been associated with increased disease susceptibility to hypertension in later life. Alterations in the maternal or fetal metabolism might impact on fetal growth and influence birth outcomes. Discerning associations between the maternal and fetal metabolome and surrogate parameters of fetal growth could give new insight into the complex relationship between intrauterine conditions, birth outcomes, and later life disease susceptibility. ***Methods:*** Using flow injection tandem mass spectrometry, targeted metabolomics was performed in serum samples obtained from 226 mother/child pairs at delivery. Associations between neonatal birth weight and concentrations of 163 maternal and fetal metabolites were analyzed. ***Results:*** After FDR adjustment using the Benjamini-Hochberg procedure lysophosphatidylcholines (LPC) 14:0, 16:1, and 18:1 were strongly positively correlated with birth weight. In a stepwise linear regression model corrected for established confounding factors of birth weight, LPC 16:1 showed the strongest independent association with birth weight (CI: 93.63 - 168.94; $P = 6.94\times10^{-11}$). The association with birth weight was stronger than classical confounding factors such as offspring sex (CI: -258.81- -61.32; $P = 0.002$) and maternal smoking during pregnancy (CI: -298.74 - -29.51; $P = 0.017$). ***Conclusions:*** After correction for multiple testing and adjustment for potential confounders, LPC 16:1 showed a very strong and independent

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association with birth weight. The underlying molecular mechanisms linking fetal LPCs with birth weight need to be addressed in future studies.

Introduction

It is by now widely accepted, that the early life environment is not just an important factor affecting immediate birth outcomes, but is also associated with cardiovascular disease susceptibility in later life – for instance the likelihood of developing hypertension. The developmental origins of health and disease (DOHaD) hypothesis, an explanatory model for the link between early life conditions and adult disease susceptibility, has been established based on the data of epidemiological [1-4] and animal studies [5-8]. As alterations in the intrauterine environment can affect fetal growth, anthropometric measures at birth, like birth weight, birth length and head circumference are well established surrogate parameters in the investigation of developmental disease origins [9]. There is a vast amount of compelling evidence in literature demonstrating that low birth weight is associated with an increased risk for metabolic and cardiovascular disease as well as hypertension in later life [2, 10-11]. Growth and development in utero are complex processes which depend on a variety of maternal, paternal and fetal factors for an optimal outcome [12]. Due to the intricacy of involved factors, our understanding of underlying mechanisms of developmental disease origins is still limited. Recent technologic advances in high-throughput methods, like array and diverse Omics approaches have revolutionized biological research. Metabolomics may capture exposures that are notoriously challenging to quantify and improve our understanding of the link between early-life environmental factors, fetal development and disorders in later life [12-14]. Targeted metabolomics can provide detailed quantitative information on the metabolic status of an organism, adding to the better characterization of phenotypes associated with metabolic and cardiovascular sequelae over the life course [14]. Metabolic profiling was already used in characterizing maternal plasma and umbilical cord blood metabolomes in conditions such as preterm birth [15], small for gestational age (SGA) [16], low birth weight [17], very low birth weight [18, 19] and intrauterine growth retardation (IUGR) [20-22]. However, a major limitation of available studies are heterogeneous study designs, applied methodology, low sample sizes, the usage of untargeted metabolomic approaches, and the lack of replication of obtained study results [15-23].

The aim of the current study was to investigate associations between the maternal and fetal metabolome at time of birth and well established birth weight in an appropriately sized mother child cohort employing a widely used targeted metabolomic approach.

Materials and Methods

Clinic data collection

This observational study was approved by the local ethics committee and carried out at the Department of Obstetrics, Charité Universitaetsmedizin Berlin (Berlin, Germany). 226 newborns and their mothers entered the study. The majority (89.9%) were of Caucasian ethnicity – for details see also [15, 24, 25].

A structured medical history was taken. The following data were extracted into our database: age, ethnicity, weight before pregnancy, body height, gravidity, parity, hypertension and diabetes mellitus during pregnancy, smoking status before and during pregnancy, systolic and diastolic blood pressure (BP) measurements recorded during pregnancy, and the mode of delivery (normal delivery or cesarean section). As well as the newborn postnatal examination biometric data birth weight, birth length, head circumference, child sex, and Apgar scores assessed at 5 and 10 minutes were collected

Sample collection

Midwives collected maternal blood from a cubital vein in the delivery room or on the ward prior to birth. Fetal blood was collected from the umbilical cord immediately after delivery. Blood was centrifuged at 2750 g and the obtained serum samples were then stored at -80 °C until it was analyzed.

Targeted metabolomics in blood serum

Metabolite quanitification were done in the Metabolomic Platform of the Genome Analysis Center, Helmholtz Zentrum München, using FIA-ESI-MS/MS and the Absolute*IDQ*™ p150 Kit (BIOCRATES Life Sciences AG, Innsbruck, Austria). The assay allows simultaneous quantification of 163 metabolites out of 10 µL serum, and includes free carnitine, 40 acylcarnitines, 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. The method of Absolute*IDQ*™ p150 Kit has been proven to be in conformance with the EMEA-Guideline "Guideline on bioanalytical method validation (July 21st 2011") [26], which implies proof of reproducibility within a given error range. A detailed description of the sample preparation, assay procedures and nomenclature have been published previously [27-29].

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.), beside standard laboratory equipment. 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 1.5.1. Data evaluation for quantification of metabolite concentrations and quality assessment was performed with the Met*IDQ*™ software package, which is an integral part of the Absolute*IDQ*™ Kit. Metabolite concentrations were calculated using internal standards and reported in µM.

Statistical analysis

Data were analyzed with SPSS version 22.0. To find associations between neonatal birth weight and targeted metabolites, a three-step analysis was used: bivariate correlation analysis, *P*-values adjustment for multiple testing, and multiple linear regression analysis. In bivariate correlation analysis, B was used to estimate the strength of a correlation. To reduce false discovery rate (FDR) due to multiple testing, resulting *P*-values from bivariate correlation analysis were adjusted using the Benjamini-Hochberg (BH) procedure. The BH procedure is defined as $P_m \leq m \times q/M$ [30, 31]. M equals the total number of tested metabolites (M=163, 163 metabolites), q equals the FDR (the FDR set up at 5% in the present paper), *P*m equals the individual *P*-value's rank, and m equals the individual rank of tested metabolite. Factors known to be associated with birth weight (gestational age [32], child sex [33], maternal age [34], maternal BMI before pregnancy [35], maternal smoking during pregnancy [36, 37]) were used as confounders to calculate and adjust putative predictive metabolites in linear regression and stepwise linear regression models. For stratifying the cohort into small (SGA), appropriate (AGA), and large for gestational age (LGA) offspring, the 10th and 97th percentiles of birth weight for the gestational age were used as cut-offs defining SGA and LGA, respectively [38, 39]. A statistically significant difference was considered as $P < 0.05$.

Results

Description of the cohort

Descriptive data of the study population, which represented a regular birth cohort in regard to key characteristics such maternal age, height, BMI before pregnancy, smoking status and newborn sex, birth weight, birth length, and head circumference, are given in Table 1. For the distribution of child birth weight see Fig. 1. A.

Bivariate correlation analyses of maternal serum metabolites and child birth weight

Bivariate correlation analyses showed that four acylcarnitines (C10:1, C14:2-OH, C16:2-OH, C18:1-OH), and one sphingolipid SM (OH) C 16:1

Table 1. Detailed Descriptive Data of the mother and child (n = 226). Data are given as mean ± SE or %

Variable	Mean±SE / %
Caucasian ethnicity/other ethnicity, %	90.0/10.0
Maternal age, y	30.4±0.4
Maternal height, cm	166.3±0.5
Maternal weight before pregnancy, kg	63.2±0.6
Maternal BMI before pregnancy, kg/m^2	22.3±0.3
Primigravida/primipara, %	37.4/62.6
Smoking before/during pregnancy, %	43.1/16.2
Hypertension before/during pregnancy, %	3.5/11.3
Diabetes mellitus before/during pregnancy, %	1.5/9.0
Mean weight 1st trimester kg	65.0±1.0
Mean weight 2nd trimester, kg	67.7±1.0
Mean weight 3rd trimester half , kg	75.2±0.0.9
Mean SBP in 1st trimester, mm Hg	113.1±1.0
Mean SBP in 2nd trimester, mm Hg	112.3±0.8
Mean SBP in 3rd trimester, mm Hg	113.3±0.7
Mean DBP in 1st trimester, mm Hg	68.7±0.7
Mean DBP in 2nd trimester, mm Hg	67.2±0.5
Mean DBP in 3rd trimester half, mm Hg	69.2±0.5
Gestational age at delivery, days	273.3±0.6
Child sex, male/female, %	52.2/47.8
Child birth weight, g	3372.3±30.5
Child birth length, cm	50.7±0.2
Child head circumference	34.7±0.1
Apgar score at 5 min	9.4±0.1
Apgar score at 10 min	9.6±0.1
Fetal cord blood pH	7.27±0.05

were positively correlated with birth weight (Table 2a). After p-value correction of the initially significant associations using the BH procedure (P_m > m×q/M), no more significant associations were observed.

Bivariate correlation analyses of fetal metabolites and child birth weight

Bivariate correlation analyses showed that nine LPCs (LPC 14:0, 16:0, 16:1, 17:0, 18:0, 18:1, 18:2, 20:3, 20:4) were positively correlated with birth weight (Table 2b). After Benjamini-Hochberg adjustment, three LPCs (LPC 14:0, 16:1, 18:1) with P_m ≤ m×q/M remained significantly correlated with birth weight (Table 2b).

Linear regression analyses of fetal metabolites and child birth weight

Significant results obtained by FDR corrected bivariate correlation analysis were subsequently analyzed by multiple linear regression analysis, considering gestational age, child sex, maternal age, maternal BMI before pregnancy, and maternal smoking during pregnancy as confounders. LPC 14:0 (Standardized beta = 0.31, $P = 1.75\times10^{-7}$), 16:1 (Standardized beta = 0.38, $P = 6.94\times10^{-11}$), and 18:1 (Standardized beta = 0.35, $P = 7.89\times10^{-9}$) were strongly correlated with birth weight (table 3a). To investigate which of the of the three identified LPCs (LPC 14:0, 16:1 and 18:1) shows the greatest impact on birth weight, a stepwise multiple linear regression model was calculated. In this model fetal LPC 16:1 (Standardized beta = 0.39, $P = 6.94\times10^{-11}$, Table 3b model B) demonstrated the strongest correlation with birth weight.

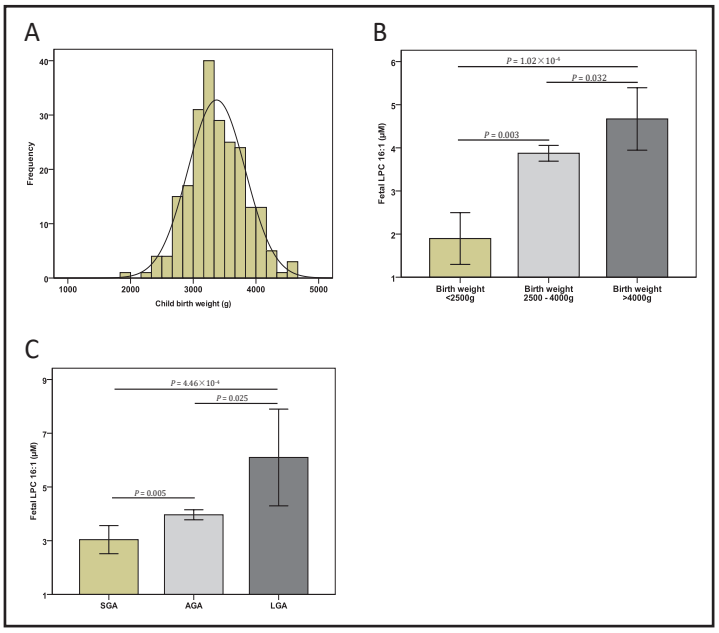

Fig. 1. Distribution of child birth weight (A), mean fetal serum LPC 16:1 in groups of child weight <2500g (n = 5), 2500-4000g (n = 199) and >4000g (n = 22) (B), and mean fetal serum LPC 16:1 in groups of SGA (n = 23), AGA (n = 198), and LGA (n = 5) (C). LPC = lysophosphatidylcholine. Data are given as mean ± SE, SGA = small for gestational age, AGA = appropriate for gestational age, LGA = large for gestational age.

Table 2. a. Correlation between maternal serum metabolites and child birth weight. (n = 226). Note: only metabolites with a p-value less than 0.05 in bivariate correlation analysis shown in the table. Cx:y = acylcarnitine, -OH = hydroxy, SM = sphingomyelin. b. Correlation between fetal serum metabolites and child birth weight. (n = 226). Note: only metabolites with a p-value less than 0.05 in bivariate correlation analysis shown in the table. LPC = lysophosphatidylcholine

a) Independent variable	Spearman Correlation	P_m value	m×q/M value
C10:1	0.144	0.031	1.23×10^{-3}
C14:2-OH	0.137	0.040	1.53×10^{-3}
C16:2-OH	0.145	0.029	9.20×10^{-4}
C18:1-OH	0.159	0.017	3.07×10^{-4}
SM (OH) C 16:1	0.156	0.019	6.13×10^{-4}
b) Independent variable	**Spearman Correlation**	**P_m value**	**m×q/M value**
LPC 14:0	0.252	2.12×10^{-4}	6.13×10^{-4}
LPC 16:0	0.223	7.42×10^{-3}	1.23×10^{-3}
LPC 16:1	0.286	2.60×10^{-5}	3.07×10^{-4}
LPC 17:0	0.200	0.003	1.53×10^{-3}
LPC 18:0	0.192	0.004	1.84×10^{-3}
LPC 18:1	0.220	8.79×10^{-4}	9.20×10^{-4}
LPC 18:2	0.139	0.037	2.76×10^{-3}
LPC 20:3	0.143	0.032	2.45×10^{-3}
LPC 20:4	0.146	0.029	2.15×10^{-3}

To rule out that the observed correlation between fetal LPC 16:1 and birth weight is an epiphenomenon mediated by hypoxic activation of phospholipase A2 and subsequent generation/ accumulation of LPCs, further statistical analyses using fetal cord blood pH and APGAR scores as parameters of birth related hypoxia were performed. Fetal LPC 16:1 was not significantly correlated to fetal cord blood pH. Furthermore, quartiles of LPC 16:1 fetal serum levels were generated and fetal cord blood pH and APGAR scores at 5 min & 10 min compared within these 4 groups. There were no significant differences in fetal cord blood pH and APGAR score at 5 min & 10 min among the LPC 16:1 quartiles (For more details, see Table 4). To further confirm that fetal LPC 16:1 is independently associated with birth weight, a third linear regression model (model C) using fetal cord blood pH and APGAR score at 5 min as confounders were conducted. Also in this model, fetal LPC 16:1 (Standardized beta = 0.39, $P = 8.17\times10^{-10}$) was clearly positively correlated with birth weight (Table 3b).

To check and confirm the final result, fetal serum LPC 16:1 concentrations were compared in low (<2500g), normal (2500-4000g) and high (>4000g) birth weight groups, and in groups small (SGA), appropriate (AGA) and large for gestational age (LGA). Newborns with low birth

Table 3. a. Linear Regression models analyzing associations between fetal serum metabolites and child birth weight. (n = 226). LPC = lysophosphatidylcholine. Model A Considering gestational age, child sex, maternal age, maternal pre-pregnancy BMI, mother smoking during pregnancy, and LPC 14:0 being the independent variable and birth weight being dependent variable. Model B Considering gestational age, child sex, maternal age, maternal pre-pregnancy BMI, mother smoking during pregnancy, and LPC 16:1 being the independent variable and birth weight being dependent variable. Model C Considering gestational age, child sex, maternal age, maternal pre-pregnancy BMI, mother smoking during pregnancy, and LPC 18:1 being the independent variable and birth weight being dependent variable. 3b. Stepwise Linear Regression models analyzing associations between fetal serum metabolites and child birth weight. (n = 226). LPC = lysophosphatidylcholine. Model A Considering gestational age, child sex, maternal age, maternal pre-pregnancy BMI, mother smoking during pregnancy being the independent variable and birth weight being dependent variable. Model B Considering gestational age, child sex, maternal age, maternal pre-pregnancy BMI, mother smoking during pregnancy, and the 3 metabolites from the above table 2b ($P_m \leq m\times q/M$) being the independent variable and birth weight being dependent variable. Model C Considering gestational age, child sex, maternal age, maternal pre-pregnancy BMI, mother smoking during pregnancy, fetal cord blood pH, apgar score at 5 min and the 3 metabolites from the above table 2b ($P_m \leq m\times q/M$) being the independent variable and birth weight being dependent variable

a	Variable	Standardized Beta	t	P	95.0% Confidence interval for B
		Model A (R^2 = 0.33)			
	LPC 14:0	0.31	5.40	1.75×10^{-7}	213.27～458.26
	Gestational age	0.42	7.32	3.62×10^{-12}	17.24～29.94
	Child sex	-0.17	-2.95	0.003	-255.75～-50.81
	Maternal age	0.15	2.59	0.010	2.78～20.56
	Maternal pre-pregnancy BMI	0.09	1.52	0.129	-2.59～20.23
	Mother smoking during pregnancy	-0.14	-2.31	0.022	-303.11～-23.90
		Model B (R^2 = 0.38)			
	LPC 16:1	0.39	6.87	6.94×10^{-11}	93.63～168.94
	Gestational age	0.47	8.30	1.22×10^{-14}	19.99～32.44
	Child sex	-0.18	-3.19	0.002	-258.81～-61.32
	Maternal age	0.14	2.52	0.013	2.37～19.53
	Maternal pre-pregnancy BMI	0.10	1.75	0.082	-1.26～20.77
	Mother smoking during pregnancy	-0.14	-2.40	0.017	-298.74～-29.51
		Model C (R^2 = 0.35)			
	LPC 18:1	0.35	6.01	7.89×10^{-9}	25.38～50.13
	Gestational age	0.47	8.07	5.30×10^{-14}	19.78～32.57
	Child sex	-0.18	-3.15	0.002	-263.23～-60.72
	Maternal age	0.16	2.79	0.006	3.62～21.10
	Maternal pre-pregnancy BMI	0.09	1.62	0.108	-2.03～20.47
	Mother smoking during pregnancy	-0.15	-2.53	0.012	-313.99～-38.96

b	Variable	Standardized Beta	t	P	95.0% Confidence interval for B
		Model A (R^2 = 0.24)			
	Gestational age	0.39	6.35	1.29×10^{-9}	14.91～28.33
	Child sex	-0.13	-2.14	0.034	-225.39～-9.12
	Maternal age	0.18	2.92	0.004	4.52～23.36
	Maternal pre-pregnancy BMI	0.06	0.96	0.337	-6.19～17.99
	Mother smoking during pregnancy	-0.15	-2.39	0.018	-328.25～-31.41
		Model B (R^2 = 0.38)			
	LPC 16:1	0.39	6.87	6.94×10^{-11}	93.63～168.94
	Gestational age	0.47	8.30	1.22×10^{-14}	19.99～32.44
	Child sex	-0.18	-3.19	0.002	-258.81～-61.32
	Maternal age	0.14	2.52	0.013	2.37～19.53
	Maternal pre-pregnancy BMI	0.10	1.75	0.082	-1.26～20.77
	Mother smoking during pregnancy	-0.14	-2.40	0.017	-298.74～-29.51
		Model C (R^2 = 0.38)			
	LPC 16:1	0.39	6.49	8.17×10^{-10}	88.56～166.31
	Gestational age	0.47	7.85	2.80×10^{-13}	19.51～32.59
	Child sex	-0.17	-2.93	0.004	-259.87～-50.93
	Maternal age	0.12	1.88	0.061	-0.42～17.93
	Maternal pre-pregnancy BMI	0.08	1.32	0.190	-3.76～18.81
	Mother smoking during pregnancy	-0.14	-2.41	0.017	-319.10～-32.10
	Fetal cord blood pH	-0.05	-0.75	0.456	-1124.26～506.98
	Apgar score at 5 min	0.16	1.82	0.070	-7.53～189.23

Table 4. Fetal cord blood pH and apgar score at 5 min & 10 min comparison according LPC 16:1 quartiles. Data are given as mean ± SE.

Variable	First quartile (n = 56)	Second quartile (n = 56)	Third quartile (n = 56)	Fourth quartile (n = 55)	P value
Fetal cord blood pH	7.25±0.01	7.25±0.01	7.29±0.01	7.27±0.01	0.072
Apgar score at 5 min	9.22±0.13	9.38±0.11	9.54±0.08	9.31±0.10	0.154
Apgar score at 10 min	9.48±0.11	9.62±0.09	9.72±0.07	9.62±0.09	0.250

weight (<2500g; n = 5) displayed significantly lower (1.90±0.21 μM) mean serum LPC 16:1 levels compared to normal (2500-4000g; n = 199; 3.85±0.09 μM) and high (>4000g; n = 22; 4.60±0.33 μM) birth weight newborns (for more details, see Fig. 1.B). Additionally, high birth weight newborns had significantly elevated serum LPC 16:1 concentrations compared to normal birth weight newborns. The same pattern of significant differences in fetal serum LPC 16:1 levels could be observed comparing SGA (n = 23; 3.04±0.25 μM), AGA (n = 198; 3.97±0.09 μM), and LGA (n = 5; 5.50±0.74 μM) newborns (for more details, see Fig. 1.C).

Discussion

The current study investigated the association between 163 maternal and fetal serum metabolites at birth and newborn birth weight in a cohort of 226 mother-newborn pairs. An initial analysis, not adjusted for confounding factors, identified associations between several maternal and fetal metabolites with birth weight. However, after FDR adjustment only fetal LPC 14:0, LPC 16:1, and LPC 18:1 were significantly associated with birth weight. Employing linear multiple regression models followed by a stepwise multiple regression model, all adjusted for confounding factors known to affect neonatal anthropometric measurements (gestational age [32], child sex [33], maternal age [34], maternal BMI before pregnancy [35], maternal smoking during pregnancy [36, 37]) revealed that out of the three LPCs, fetal serum LPC 16:1 showed the strongest independent association with birth weight. Data stratification into groups of birth weight (<2500g; 2500-4000g; >4000g;) and groups of size for gestational age (SGA; AGA; LGA) further substantiated the positive association between fetal LPC 16:1 and size at birth.

One possible explanation of the observed positive association between fetal LPC 16:1 and birth weight might be hypoxia. It is well known that hypoxia activates phospholipases that induce the generation of LPCs [40-42]. The process of giving birth is characterized by hypoxic periods of varying duration for both, the mother and the fetus. Theoretically, due to a longer duration of giving birth, larger newborns may be subjected to longer acute periods of hypoxia and thus display a stronger activation of phospholipases and higher levels of LPCs. However, in the current study we were not able to observe a significant association between data indicative of hypoxia, ie. fetal cord blood pH and APGAR scores, and fetal LPC 16:1 levels.

To the best of our knowledge, this is the second large scale study showing an independent association between fetal LPCs and birth weight. Very recently, Hellmuth et al. demonstrated in a large birth cohort a strong independent positive correlation between birth weight and several cord blood LPCs, including LPC 14.0, LPC 16:1, and LPC 18:1 [43]. Similarly, also in the current study these metabolites were independently associated with birth weight after FDR adjustment. Furthermore, also in the study by Hellmuth et al. LPC 16:1 demonstrated the strongest association with birth weight. In addition, several metabolites were negatively associated with birth weight, which might have been due to the larger cohort size of 753 fetal cord blood samples/newborns. Different from the current study, Hellmuth et al. did not use a commercially available kit for the metabolomic analyses but several methodological approaches targeting different classes of metabolites. Based on these methodological differences absolute metabolite concentrations might not be comparable between the two studies. The general tendencies of metabolite concentrations i.e. in relation to birth weight should not be affected. This underscores a putatively important role of specific LPCs in fetal growth. Based on the current state of literature, however, not much is known regarding

underlying mechanisms of the observed association. LPCs in the adult organism result from partial hydrolysis of PCs by the enzymatic action of phospholipase A2 [44], from hepatic secretion [45], and lecithin-cholesterol acyltransferase [46]. Several studies have linked LPCs with obesity, but results are contradictive, which might be due to the multifaceted incompletely understood functions of LPCs [47-49]. Higher levels of LPC species were reported in obese men [50] and also observed in an obese monozygotic twin study [51]. In a study designed to discover biomarkers that are indicative of weight change, LPC 14:0 was shown to be strongly positively associated with rapid growth and childhood obesity [52]. In contrast to these positive associations other studies showed negative associations between serum LPCs and obesity [53-57]. Overweight/obese children had decreased levels of the unsaturated LPC 18:1 [53, 54] compared with normal weight children. The comparison of adult overweight/obese subjects to their lean counterparts demonstrated increased concentrations of saturated LPCs (LPC 14:0, 18:0) and decreased concentrations of the unsaturated LPC 18:1 [55]. Also in an animal model of obesity decreased levels of the unsaturated LPCs 16:1 and 18:1 were observed [58]. Given the inhomogeneity of study results and lack of available suitable mechanistic data, it is hard to draw any firm conclusions on how LPCs might influence body weight, especially in the fetal organism. However, one mechanism that is believed to negatively affect offspring birth weight that might also be connected to LPC metabolism is fetal insulin resistance. Insulin is one of the main culprits of macrosomia in children born to diabetic mothers [59]. Contrary, attenuated fetal insulin signaling, as found in insulin resistance, has been suggested to decrease birth weight and to be a characteristic of the low birth weight phenotype [60, 61]. It was shown, that certain LPCs (among these LPC14:0) can interact with glucose metabolism independent of insulin signaling and lead to enhanced cellular glucose uptake [62]. Transcriptome analyses of human myotubes treated with LPC 16:0 and 18:1, demonstrated an increased expression of PPARδ regulated transcripts, inducing anti-diabetic and antiinflammatory effects [63]. Furthermore, it was demonstrated that LPCs (especially LPC 18:1) can enhance glucose-dependent insulin secretion in perfused rat pancreas via an orphan G-protein coupled receptor [64]. A study investigating associations between BMI, inflammation and insulin resistance demonstrated negative associations between LPC 18:1 and various adipokines and inflammatory mediators. Low levels of LPC 18:1 together with increased levels of leptin or CRP were associated with increased HOMA scores [65]. Applied to the results of the current study, altered levels of LPCs hypothetically could affect fetal growth by influencing insulin resistance and insulin secretion. Lower levels of LPCs, as found in offspring with lower birth weight, could result in increased fetal insulin resistance and decreased insulin secretion, which could negatively affect fetal growth. This hypothesis is interesting in context with previous findings from our group. In two independent previous studies we observed a negative correlation between total glycated cord blood hemoglobin and birth weight, an observation that contrasts the usual positive correlation between maternal glycemia and birth weight [66, 67]. Results of these studies indicated that lighter fetuses, when subjected to similar degrees of maternal glycemia, display an incapability of adequately lowering their blood glucose concentrations (reflected by elevated cord blood total glycated hemoglobin), in comparison to heavier fetuses. The mechanism behind these associations remained unexplored, but alterations in LPC metabolism may serve as a link in the connection between impaired fetal glucose handling and low birth weight.

The reason why LPCs 14:0, 16:1 and 18:1 were lower in the serum of lower birth weight newborns cannot be answered by our study. Several factors including maternal dietary intake, placental transfer and fetal production are possible. In the current study, levels of LPC 16:1 were about threefold higher in fetal compared to maternal serum, yet showed a barely significant positive correlation (data not shown). This finding suggests that LPC 16:1 might predominantly be generated in the fetus, but does not preclude alterations in the trans-placental transport of precursor forms. Interesting in this regard, LPC 16:0 concentrations did not differ between mother and fetus and there was a modest, highly significant correlation between fetal and maternal LPC 16:0 levels. The same pattern of absent maternal-fetal

concentration differences and presence of significant correlations could be observed for the saturated LPCs 14:0 and 18:0.

Study limitations and Outlook

One limitation of the current study is that serum metabolites were only measured at one occasion prior to birth. Furthermore, we cannot rule out if specific phenotypic and lifestyle factors which we did not account for, may have influenced the results. However, we used a very strict approach, employing BH procedure to reduce false discovery rate, followed by linear regression analysis adjusted for confounding factors known to affect neonatal anthropometric measurements. Moreover, the results of the study remain purely associative. Future studies are needed to investigate underlying mechanisms of the observed associations. Despite of the mentioned study limitations, a strength of the current study is the replication of major findings of a previous study by Hellmuth et al., [43] which is a crucial aspect of research based on high-throughput data.

In conclusion, after correction for multiple testing and adjustment for potential confounders, lysophosphatidylcholine 16:1 showed a very strong and independent association with birth weight, a surrogate parameter of intrauterine development and adult disease susceptibility. In the future, suitable preclinical studies are needed to better characterize underlying mechanisms of the observed association and to investigate if there is a mechanistic link between low birth weight, insulin resistance and alterations in LPC metabolism. Future clinical studies should include additional collection of information on possible 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.

Conclusion

The aim of the current study was to investigate associations between the maternal and fetal metabolites and birth weight. There were no correlations between maternal metabolomics and birth weight. After correction for multiple testing and adjustment for potential confounders, LPC 16:1 showed a very strong and independent association with birth weight. This correlation was even stronger than classical confounding factors such as maternal smoking orr offspring sex.

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Disclosure Statement

No conflict of interest exists.

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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

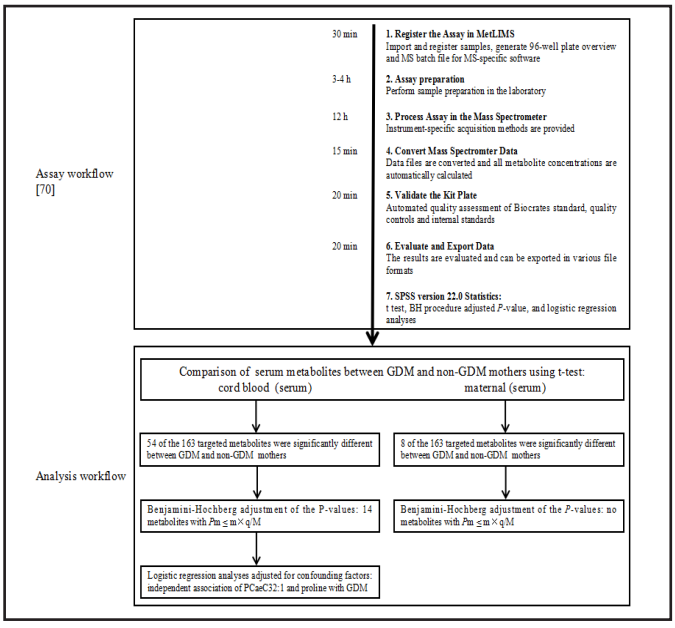

Fig. 1. Flowchart for the metabolomics analysis strategy. M = total number of analyzed metabolites (M=163); q = FDR; m = the individual rank of tested metabolite; Pm = the individual P-value; PC = phosphatidylcholine; ae = acyl-alkyl. The Assay Workflow was adapted from: AbsoluteIDQ® p150 Kit - Biocrates. 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	$1.44\times10^{-2}\pm3.73\times10^{-3}$	$1.64\times10^{-2}4.69\times10^{-3}$	-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}

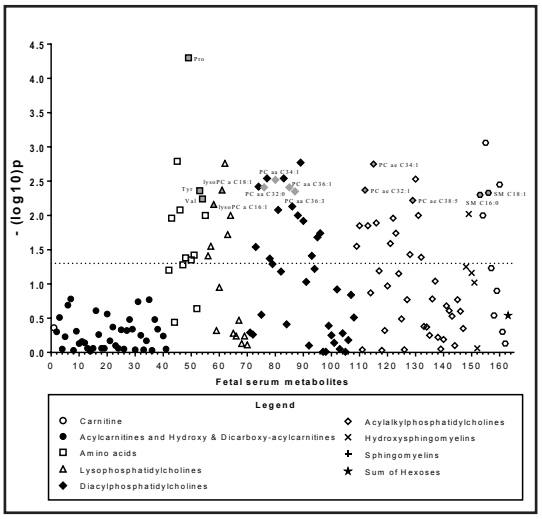

Fig. 2. Manhattan Plot of all fetal serum metabolites. The –log10 of resulted p-values is shown. The level of significance for the unadjusted analyses is shown by a dotted line. Each class of metabolites is marked with a respective symbol. Metabolites shown in grey together with the respective metabolite 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

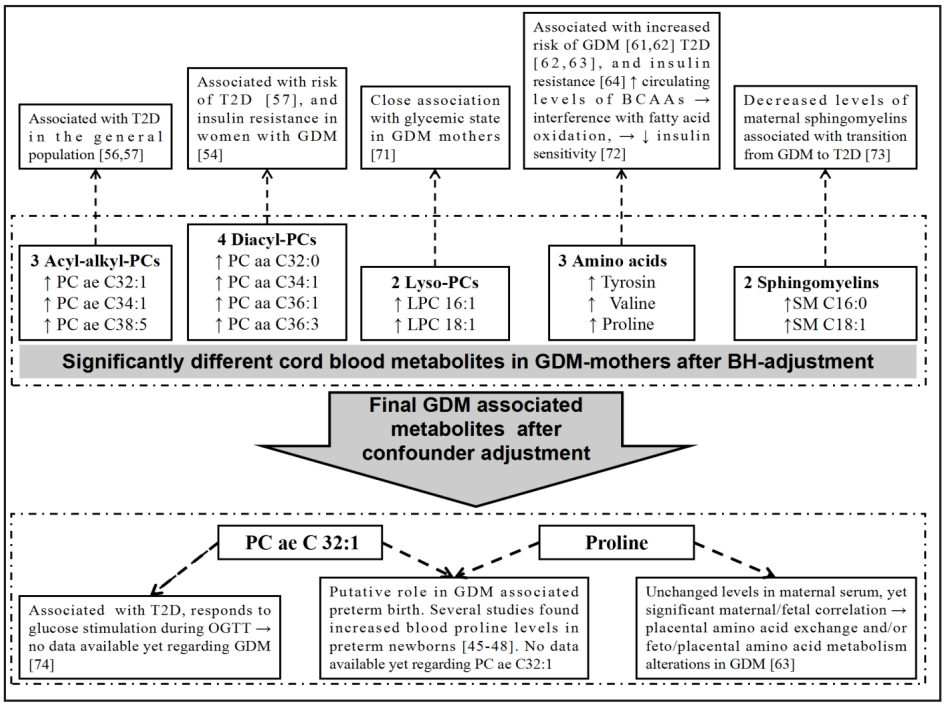

Fig. 3. Pathway of relevant metabolites for GDM. PC = phosphatidylcholine; ae = acyl-alkyl; SM = Sphingomyelins; OH = hydroxy; BCAAs = Branched-chain amino acids.

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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Curriculum Vitae

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

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