Pediatric RESEARCH

CLINICAL RESEARCH ARTICLE

Umbilical cord blood metabolomics: association with intrauterine hyperglycemia

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BACKGROUND: Intrauterine hyperglycemia can harm a fetus's growth and development, and this can be seen in the umbilical cord blood metabolism disorder. However, the metabolites and metabolic mechanisms involved in the condition remain unknown.
METHODS: Targeted metabolomics using liquid chromatography and MetaboAnalyst were conducted in this study to explore differences in metabolites and metabolic pathways between individuals with hyperglycemia or well-controlled gestational diabetes mellitus (GDM) and healthy controls.
RESULTS: Univariate analysis found that the hyperglycemic and healthy control groups differed in 30 metabolites, while the well-controlled GDM and the healthy control groups differed only in three metabolites—ursodeoxycholic acid, docosahexaenoic acid, and 8,11,14-eicosatrienoic acid. Most of these metabolic variations were negatively associated with neonatal weights. Further research showed that the variations in the metabolites were primarily associated with the metabolic pathways of linoleic acid (LA) and alpha-linolenic acid (ALA).
CONCLUSION: Gestational hyperglycemia and well-controlled GDM, which may play a major role by inhibiting the LA and ALA metabolic pathways, have detrimental effects on cord blood metabolism.

Pediatric Research (2022) 91:1530–1535; https://doi.org/10.1038/s41390-021-01516-4

IMPACT:

- The main point of this paper is that intrauterine hyperglycemia has a negative effect on cord blood metabolism mainly through the linoleic acid and alpha-linolenic acid metabolic pathways.
- This is a study to report a new association between well-controlled GDM and cord blood metabolism.
- This study provides a possible explanation for the association between intrauterine hyperglycemia and neonatal adverse birth outcomes.

INTRODUCTION

Gestational diabetes mellitus (GDM) is historically characterized as any irregular glucose metabolism with onset or first detection during pregnancy and is typically diagnosed within the second trimester. Intrauterine hyperglycemia not only endangers the health of the mother but also has a detrimental effect on the perinatal outcome and even the development of the offspring.[1–3] While treatments can reverse hyperglycemia and reduce the risk of the associated adverse pregnancy outcomes,[4] well-controlled GDM during pregnancy continues to have negative effects on the child's long-term growth.[5] It is therefore important to elucidates the mechanism by which intrauterine hyperglycemia affects the development of the fetus.

Metabolomics, the systematic study of metabolites found in the blood and other tissues, provides an approach to the identification and understanding metabolic signatures involved in intrauterine hyperglycemia and associated metabolic disorders, such as GDM. Multiple studies have indicated that the mother's metabolic status will affect the levels of different metabolites and the metabolic homeostasis of the fetus, which is associated with an increased risk of macrosomia and respiratory distress syndrome.[2,3] The umbilical cord serves as a bridge for the exchange of materials between the mother and the child, and cord blood metabolites may reflect the metabolic state of the fetus. Existing research focuses mainly on several essential polyunsaturated fatty acids, such as docosahexaenoic acid (DHA) and arachidonic acid (AA).[6,7] The function of other metabolites in maternal hyperglycemia-related risk is unknown, and research on the relationship between well-controlled GDM and cord blood metabolites are limited. Targeted metabolomic tests are therefore conducted on 86 cord blood samples to investigate the effects of hyperglycemia and well-controlled GDM on cord blood metabolism during pregnancy.

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Received: 23 December 2020 Revised: 11 March 2021 Accepted: 20 March 2021
Published online: 12 May 2021

MATERIALS AND METHODS

Chemicals and reagents

The main instruments used in this study were as follows: Agilent 1290 UHPLC system (Agilent Technologies, Santa Clara, CA, USA) and ultrapure water preparation system (produced by Millipore, Billerica, MA, USA). The internal standard 12(S)-HETE-d8 was provided by Cayman Chemical (Ann Arbor, MI, USA). 1-Hydroxybenzotriazole hydrate (HOBt), 1-(bis(dimethylamino) methylene)-1H-1,2,3-triazolo(4,5-b) pyridinium 3-oxid hexafluorophosphate (HATU), and triethylamine were purchased from Sigma-Aldrich Laboratories, Inc. (St. Louis, MO, USA). Cholamine has been obtained from Santa Cruz Biotechnology, Inc. (Indian Gulch, CA, USA). Acetonitrile (MS grade), dimethyl sulfoxide (DMSO, MS grade), and methanol (ACS grade) were supplied by J.T. Baker (Danville, PA, USA).

Sample collection

A total of 86 pregnant women who delivered their newborns at the Obstetrics Department of the First Affiliated Hospital of Guangzhou Medical University were invited to participate. Umbilical cord blood was collected by needle aspiration of the umbilical artery within 5 min of birth and prior to delivery of the placenta. The samples were processed by centrifugation at 1200 × *g* for 5 min, after which the serum was collected and frozen at −80 °C prior to metabolomic assays. GDM was diagnosed according to guidelines of the American Diabetes Association. Outpatient examination information, such as age, body mass index (BMI), and other clinical data, was collected, and statistical analysis was performed as shown in Table 1. All research participants obtained informed consent, and the design of the study was approved by the ethics committee of the First Affiliated Hospital of Guangzhou Medical University (Reference number: GYFYY-2016-73).

Table 1. Baseline characteristics of study populations.

	Hyperglycemia ($n = 40$)	Well-controlled GDM ($n = 10$)	Healthy control ($n = 37$)
Maternal characteristics, mean (quartile)			
Age	30.5 (8)	29 (7.75)	30 (4.5)
Fasting plasma glucose, mg/dL	6.16 (1.09)	4.44 (0.69)	4.38 (0.57)
Pre-pregnancy BMI, kg/m^2	20.78 (2.47)	20.2 (2.88)	19.57 (3.18)
Prenatal BMI, kg/m^2	26.23 (3.36)	25.44 (5.22)	25.07 (3.63)
Gestational age, weeks	39 (1)	38 (1.5)	39 (1)
Newborn characteristics, mean (quartile)			
Sex, *N* (%)			
Male	21 (54)	7 (70)	20 (54)
Female	18 (46)	3 (30)	17 (46)
Birth weight, kg	3.34 (0.43)[a,*]	3.1 (0.37)	3.21 (0.65)[a,*]
Size for age, N (%)			
SAG	2 (5)	1 (10)	8 (22)
AGA	37 (95)	9 (90)	28 (75)
LGA	0	0	1 (3)

LAG large for gestational age, *AGA* appropriate for gestational age, *SGA* small for gestational age.
*$P < 0.05$.
[a]There were statistically significant differences between the hyperglycemia group and the healthy control group.

Derivatization

Serum samples were thawed at room temperature and mixed by shaking. The internal standard (HETE-d8) was added to 50 µL serum. Samples were then extracted with 200 µL precooled methanol for three times and then dried with nitrogen for about 30 min. Light-yellow crystals or thin films were observable. Next, we mixed 5 µL of 20 mM HOBt in DMSO, 5 µL of 100 mM cholamine in DMSO, 5 µL of 20 mM HATU in DMSO, and then incubated at room temperature for 1 min. Finally, 35 µL acetonitrile was added to the samples to ensure a final volume of 50 µL.

Chromatographic and mass spectrometry conditions

A Waters HSS T3 column (2.1 × 100 mm, 1.8 µm) was used to separate the serum metabolic profile on an Agilent 1290 UHPLC system. Chromatographic separation was performed using a mobile phase consisting of water (A) and acetonitrile (B), each containing 0.1% formic acid. The LC gradient is shown in Supplementary Table 1. The column temperature was held at 30 °C, the autosampler was set at 4 °C, and the flow rate was 0.3 mL/min.

Mass spectrometry analysis was performed on the Agilent 6545 UHD accurate mass Q-TOF/MS system with a dual-jet electrospray ion source. The instrument was operated in positive full scan mode, the mass spectra were recorded in the range of 200–1000 *m/z*, and all peaks were accurately measured. The parameters of the jet stream technology include a superheated nitrogen sheath-gas temperature of 300 °C and a flow rate of 11 L/min. The MS parameters were as follows: drying gas flow rate, 11 L/min; gas temperature, 250 °C; nebulizer pressure, 22 psi; capillary voltage, 3500 V; nozzle voltage, 500 V; and fragmentation voltage, 175 V. Atomizing a low-flow TOF reference mixture for continuous calibration in cation mode: 922.0098 (C18H18F24N3O6P3).

Data processing and statistical analysis

The experimental data adopted Qualitative Analysis B.05.00 to determine the peak area. MetaboAnalyst software was used to perform preprocessing, such as missing-value filtering and filling and data standardization. SPSS 25.0 was used for univariate statistical analysis. The Mann–Whitney *U* test and Spearman tests were used because most of the metabolic data were not normally distributed. The level of significance was set at $P < 0.05$. Human endogenous metabolites were identified and the metabolic pathways were analyzed using the Human Metabolome Database and MetaboAnalyst biobases.

RESULTS

Demographic and clinical indicators

The GDM group was the patients who underwent oral glucose tolerance test (OGTT) in the second trimester and were diagnosed as GDM by the clinician; the hyperglycemia group referred to the pregnant women with normal OGTT in the second trimester and the fasting blood glucose ≥5.1 mg/dL in the third trimester in this study; the well-controlled GDM group was defined as the pregnant women who were diagnosed with GDM by OGTT in the second trimester and had good blood glucose control in the third trimester; the healthy control group was included pregnant women with constantly optimal blood glucose levels. However, since there was no statistical difference between the GDM group and the hyperglycemia group, the two groups were generally called the hyperglycemia group.

After standardization of the umbilical cord blood metabolism results, we performed a group-specific difference analysis of 85 metabolites. We found no significant difference between the GDM and the hyperglycemic groups. Therefore, as long as there is abnormal blood glucose in the third trimester, umbilical cord blood will have similar metabolism characteristics, regardless of whether the patient has been diagnosed with GDM. However,

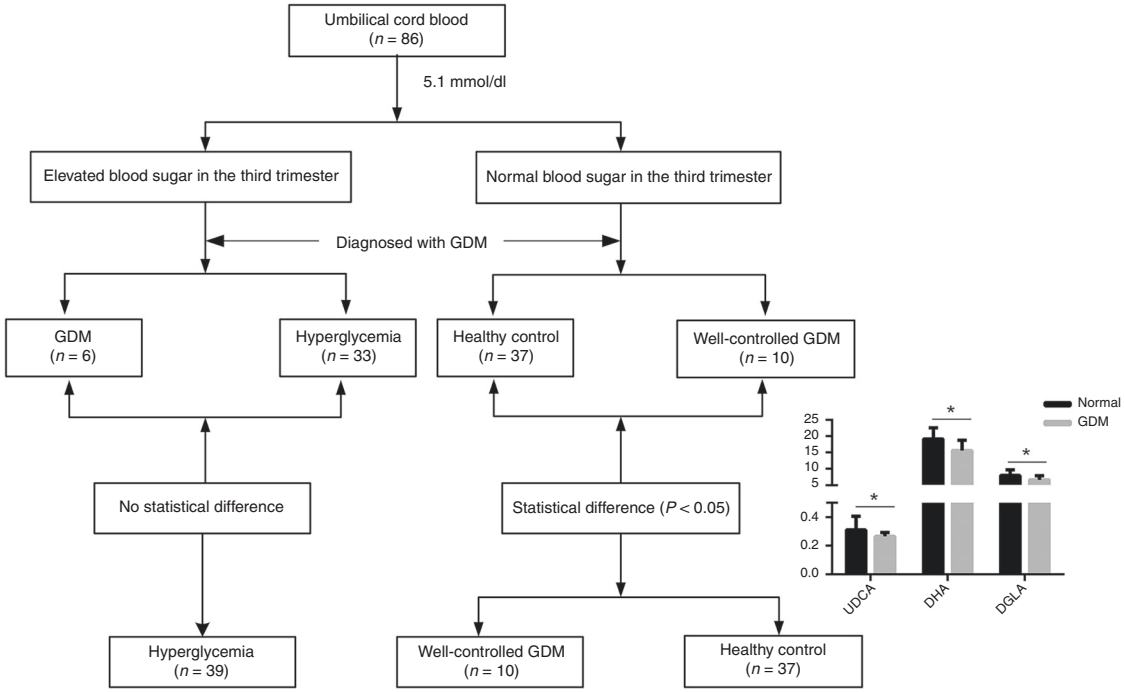

Fig. 1 Participant disposition. GDM gestational diabetes mellitus, UDCA ursodeoxycholic acid, DHA docosahexaenoic acid, DGLA 8,11,14-eicosatrienoic acid.

statistically significant differences were observed between the well-controlled GDM group and healthy control group (Fig. 1). Therefore, the well-controlled GDM group cannot be considered as having similar metabolic characteristics as the healthy control group.

After the above procedure, 86 patients were classified into three groups: the hyperglycemic group, the well-controlled GDM group, and the healthy control group. There were no statistically significant differences in age, gestational age, pre-pregnancy BMI, size for age (Table 1), among the three groups.

Univariate and correlation analysis

Eighty-five metabolites, associated with lipid metabolism, sugar metabolism, amino acid metabolism, etc., were detected in serum by a targeted cholamine derivatization-UHPLC-Q-TOF/MS approach. Cluster analysis (Fig. 2a) shows that metabolites in the hyperglycemic group and the well-controlled GDM group were significantly lower than those in the healthy control group. Compared to the healthy control group, 30 differential metabolites were found in the hyperglycemic group (Supplementary Table 2), while only three differential metabolites were found in the well-controlled GDM group: ursodeoxycholic acid, DHA, and 8,11,14-eicosatrienoic acid (DGLA) (Supplementary Table 2, among the 30). There were differences in the levels of caprylic acid, capric acid, and tetracosanoic acid (Supplementary Table 2) between the hyperglycemic group and the well-controlled GDM group. This also showed that umbilical cord blood metabolism in the well-controlled GDM group did not return to normal (Fig. 2b). The analysis of umbilical cord blood metabolites showed that there was no difference in the size for age between the hyperglycemia group and the well-controlled GDM group, while compared with the hyperglycemia group, metabolic differences of 9(S)-HPODE, caproic acid, proline, and asparagine were observed in the healthy control group.

We found that the infant weight of the hyperglycemic group was significantly higher than that of the healthy control group, and the difference was statistically significant ($P < 0.05$, Table 1). And fasting blood glucose of all pregnant women in late pregnancy correlated with infant weight to a certain extent ($r = 0.258$, $P < 0.05$, Table 1). Studies have shown that cord blood metabolism is associated with neonatal birth outcome and growth pattern. Therefore, we analyzed the association between differential metabolites and neonatal weight after adjusting for maternal age, pre-pregnancy BMI, prenatal BMI, gestational age, and fasting plasma glucose. The results show that most of the metabolites in umbilical cord blood were negatively correlated with neonatal body weight, and the six metabolites with statistically significant differences (Supplementary Table 2) were negatively correlated with neonatal body weight. DHA was also negatively correlated with neonatal weight in the well-controlled GDM group compared with that in the control group.

Metabolic pathways

Metabolite sets enrichment overview indicated that identified metabolites were primarily involved in three pathways and that the most enriched networks were LA and ALA metabolic pathways (Fig. 3a, b; Impact = 0.56), involving AA, ALA, gamma-linolenic acid, DHA, eicosadienoic acid, adrenic acid, and DGLA (Fig. 3c). In contrast, two metabolites (DHA and DGLA) of the three metabolites in the well-controlled GDM group were distributed in the LA and ALA metabolic pathways (Impact = 0.11). Thus, the metabolism did not return to normal in well-controlled GDM group in the third trimester following care. Analysis found that the metabolic pathways of LA and ALA in umbilical cord blood were most affected by maternal blood glucose.

DISCUSSION

This study demonstrates associations of cord blood metabolites with maternal blood glucose in late pregnancy. Studies have shown that there are changes in various metabolic pathways in serum of pregnant women with GDM, such as fatty acid metabolism, butyric acid metabolism, bile secretion, amino acid metabolism, etc.,[8] which is similar to the metabolic trend of placenta, but different from that of newborn.[6] Our research further proves that there are differences in umbilical cord blood metabolism in the hyperglycemia group, the well-controlled

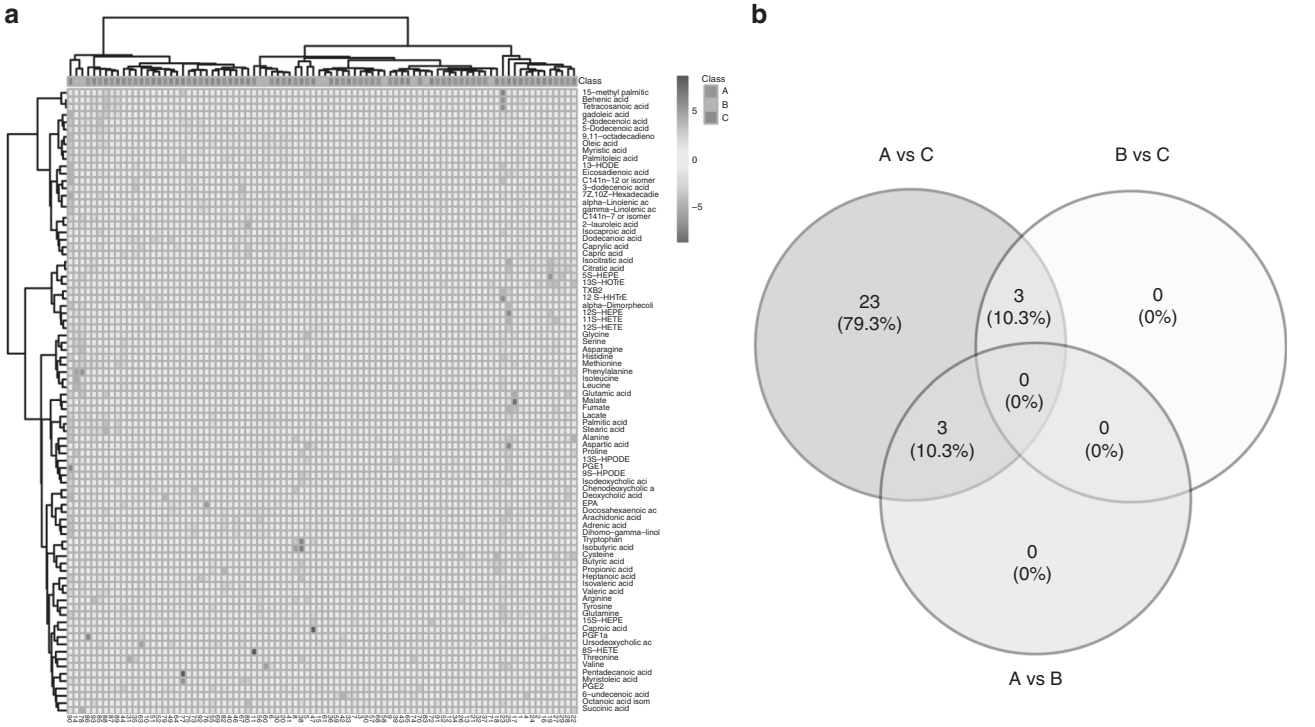

Fig. 2 Analysis of metabolites. a Clustering distribution diagram of metabolites in the three groups: A, the hyperglycemia group; B, the well-controlled GDM group; and C, the healthy control group. **b** The distribution diagram of metabolites in the three groups compared with metabolites in pairs.

GDM group and the healthy control group in the third trimester. We report novel associations of cord metabolites with the well-controlled GDM while also demonstrating that LA and ALA metabolic pathways are the key metabolic pathway through which maternal hyperglycemia affects the metabolic homeostasis of cord blood.

GDM is well known to be a common medical complication during pregnancy and harmful to mothers and fetuses. Pregnancy requires metabolic, hormonal, and immune communication between the mother and the fetus.[9] Therefore, the metabolic abnormality of pregnant women is likely to affect the metabolic characteristics of the fetus. Our study further found that not only GDM, but also well-controlled GDM and hyperglycemia patients could affect the homeostasis of cord blood metabolism. Genetic studies have shown that genome-wide methylation of fetal cord blood in pregnant women with GDM is significantly different from that in normal pregnant women, and this could have an effect on the epigenetic characteristics of offspring.[10,11] Other studies have shown that the methylation of umbilical cord blood in pregnant women with GDM following treatment is still abnormal[12] and has adverse effects on the long-term development of fetus.[5] This could explain why treatment can maintain blood glucose at a good level in pregnant women, and good control of GDM in the third trimester can still have a negative effect on the metabolic status of the fetus after birth.

In the middle and late gestation, GDM lipodecomposition and gluconeogenesis are further enhanced, leading to an increase in maternal serum non-esterified fatty acids (NEFA, including palmitic acid, oleic acid and linoleic acid, etc.).[11,13] Studies have shown that maternal NEFA level is positively correlated with neonatal weight and fat mass.[14,15] In NEFA, major omega-6 (n-6) and n-3 polyunsaturated fatty acids LA and ALA and their derivatives are involved in the development of GDM[16] and fetal growth and development.[17] Among them, AA, EPA, and DHA, especially DHA, are crucial for the development of fetal brain and retina.[18–20] We observed that the levels of oleic acid, linoleic acid, linolenic acid, and their derivatives in umbilical blood of pregnant women with hyperglycemia decreased compared to those of healthy people, which was consistent with previous studies,[21] indicating that hyperglycemia during pregnancy increased fetal lipid utilization and promoted fat accumulation. Obviously, not all differential metabolites show a downward trend, and our study found elevated levels of the metabolite tetracosanoic acid in cord blood. Of the many adverse birth outcomes, cardiovascular disease is one of the most severe and has a long-term impact on the development of newborns.[22] Tetradecanoic acid has been shown to be negatively associated with cardiovascular health,[23] but more research is required to establish if tetradecanoic acid is involved in the pathogenic mechanisms of cardiovascular disease.

In addition to the polyunsaturated fatty acids mentioned above, saturated fatty acids such as caproic acid, octanoic acid, and lauric acid are associated with lipid metabolism and glucose homeostasis.[24,25] Saturated fatty acids may predict the development of GDM in early pregnancy,[24] but the effect of maternal hyperglycemia on neonatal saturated fatty acids remains unclear. Our study found that the offspring of hyperglycemic mothers had lower saturated fatty acids, while the offspring of obese mothers also had lower saturated fatty acids.[26] Fatty acid biosynthesis has been shown to increase in pregnant women with GDM,[27] which promotes lipid accumulation in both the mother and the fetus. By analyzing metabolic pathways, our study found that most abnormal saturated fatty acids were involved in fatty acid biosynthesis pathways. This provides a possible explanation for the negative correlation between umbilical cord blood saturated fatty acids and neonatal weight.

Tyrosine is a form of aromatic amino acid (AAAs), also referred to as the human body essential amino acid. Studies have shown that tyrosine can predict the incidence of GDM during pregnancy[28] and that it is associated with neonatal weight.[14] Our research showed that offspring of hyperglycemic mothers had lower levels of tyrosine and were positively correlated with newborn weight, which is consistent with previous studies.[29]

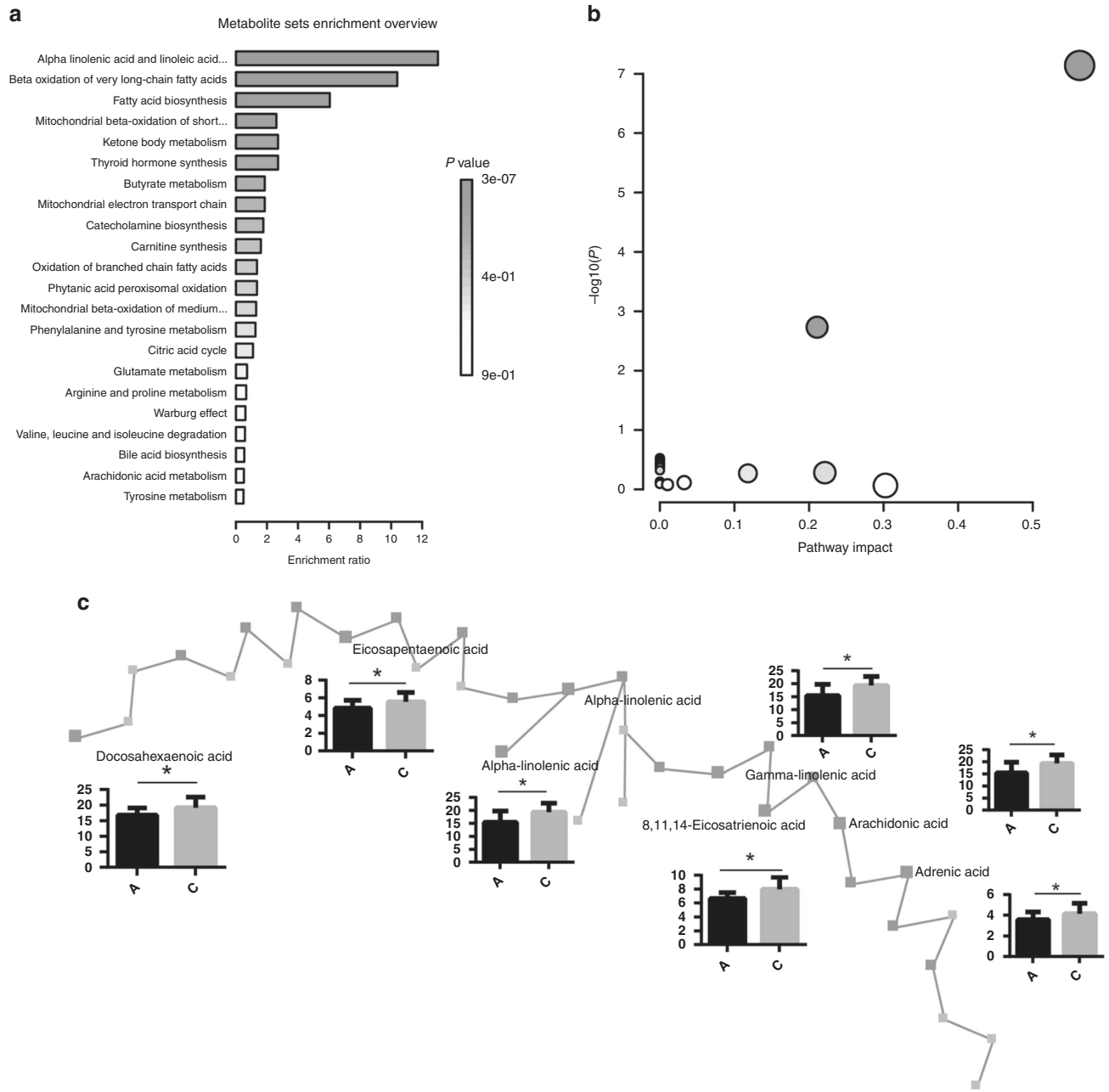

Fig. 3 Analysis of metabolic pathways. a Metabolite sets enrichment overview. **b** Metabolome view, showing all matched paths according to the *P* value in path enrichment analysis and the path impact value in path topology analysis. **c** LA and ALA metabolic pathways, with blue dots representing metabolites and gray dots representing proteases required for the reaction, and red dots are the metabolites that exist in this study.

Other AAAs and branched-chain amino acids in umbilical cord blood are associated with the birth status of newborns,[29] but no abnormalities of these amino acids have been observed in the offspring of hyperglycemic pregnant women.

Succinic acid, an essential intermediate in the tricarboxylic acid cycle (TCA), plays a key role in the production of adenosine triphosphate and has recently been shown to act as an inflammatory signal.[30] Studies have shown that during pregnancy, the concentration of TCA intermediates rises with an increase in pregnancy,[31] whereas the level of TCA metabolites is higher in diabetic patients.[32] There is no clear analysis of changes in the succinic acid content in the GDM progeny, but we observed for the first time that succinic acid content decreased in the progeny of hyperglycemic mothers and was negatively correlated with neonatal weight.

Blood glucose concentration during pregnancy negatively affects post-birth child growth patterns and weight changes[2,3] and may even affect the child's behavior and intellectual development; these effects are negatively correlated with maternal blood glucose control.[33] Our study also found a positive correlation between neonatal weight and maternal late glucose levels, consistent with existing studies. From the metabolism point of view, the difference in metabolite level in the hyperglycemic group was negatively correlated with neonatal weight. Interestingly, DHA levels were lower than average in both the hyperglycemic group and the well-controlled GDM group. After adjustment of maternal age, pre-pregnancy BMI, prenatal BMI, and gestational age, DHA was always negatively correlated with neonatal weight. It is therefore reasonable to believe that DHA plays a role in regulating neonatal weight.

Only 85 metabolites were targeted due to methodological limitations and most of which were present at low concentrations. However, due to the lack of targeting in underived metabolomics, some common and high content metabolites are often analyzed, resulting in differential metabolites that are not disease-specific. In our study, the relatively mature DIAAA derivation method was used to process the samples and detect low-level carboxylic metabolites and important physiological functions, and a univariate analysis was performed in combination with clinical data, making the differential metabolites obtained more reliable. The main strength of this research, on the other hand, is the discovery, through a thorough review of the metabolic pathways, of a novel link between well-controlled GDM and cord blood metabolism. These differential metabolites and associated differential metabolic pathways offered some guidance to understand GDM pathogenesis. On the other hand, the aim of this study was to investigate the relationship between pregnancy hyperglycemia and cord blood metabolism, while the relevant information post-birth neonates is not complete and further studies are required.

AUTHOR CONTRIBUTIONS

J.M. and J.L. made substantial contributions to the design and conception, data analysis and interpretation, drafting the manuscript, and critical revision of the manuscript. M.H., J.L., and Y.D. were included in acquisition of the data. X.B. did the interpretation of the data. B.S. did the critical analysis of the manuscript. H.C. did the critical analysis and revision of the manuscript. All authors read and approved the final manuscript.

FUNDING

This work was supported by the National Natural Science Foundation of China (grant number 81802076, 81871736); National Key Technology R&D Program (grant number 2018YFC1311900); Guangdong Science and Technology Foundation (grant number 2019B030316028); Guangzhou Municipal Health Foundation (grant number 20191A011073); and Guangzhou Science and Technology Foundation (grant number 201804020043).

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41390-021-01516-4.

Competing interests: The authors declare no competing interests.

Ethics approval and consent to participate: The study protocol was approved by the Ethics Committee of the First Affiliated Hospital, Guangzhou Medical University (Reference number: GYFYY-2016-73). Written informed consent was obtained from the volunteer before they participated in this study.

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