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Original article

Gestational diabetes mellitus-associated changes in the breast milk metabolome alters the neonatal growth trajectory

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SUMMARY

Background: Gestational diabetes mellitus (GDM) is the most common metabolic disturbance during pregnancy and leads to an altered metabolic profile of human breast milk (HBM). The association between HBM metabolites and neonatal growth in GDM pregnancies has not been thoroughly investigated.
Aims: The primary aim was to quantify differences in the HBM metabolome between normal and GDM pregnancies. The secondary aim was to identify metabolites associated with neonatal growth during the first year postpartum.
Methods: In the present study, mothers intending to exclusively breastfeed (BF) and their newborns (mother–infant pairs) were recruited at delivery (n = 129 normal pregnancies and n = 98 GDM pregnancies). HBM samples (colostrum, transition milk, and mature milk) from mothers with normal pregnancies (n = 50) and GDM pregnancies (n = 50) were subjected to metabolomic profiling via liquid chromatography tandem mass spectrometry (LC-MS/MS). Receiver operating characteristic (ROC) analysis revealed the metabolomic fingerprints of GDM-associated mature HBM. Correlations between metabolites and neonatal body weight gain (BWG) were evaluated by Spearman correlation analysis.
Results: In total, 620 metabolites were identified in each HBM sample; 253 compounds had the same variation patterns, whereas 38 compounds had significantly different pattern transitions between the GDM and normal groups. Moreover, 12, 49 and 28 metabolites exhibited significant differences in the 3 milk types between the 2 groups. Twenty-two metabolites were confirmed by ROC analysis as metabolomic fingerprints in the mature BM of GDM patients. Ten compounds were significantly negatively correlated with neonatal growth, and only 2 unsaturated lipids (eicosatrienoic acid (FA 20:3) and lysophosphatidylcholine (LysoPC) (22:6)) were positively correlated with neonatal BWG.
Conclusions: GDM is associated with alterations in the HBM metabolome. Only a small subset of compounds are associated with neonatal body weight (BW).

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Abbreviations

AA	Amino acids
BL	Body length
BWG	Body weight gain
BW	Body weight
BF	Breast feeding
d	Day
FDR	False discovery rate
FC	Fold change
QC	Quality control
GC-MS	Gas chromatography-mass spectrometry
GDM	Gestational Diabetes Mellitus
HC	Head circumference
HBM/BM	Human breast milk/Breast milk
HMO	Human milk oligosaccharide
IQR	Inter quartile range
LGA	Large for gestational age
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LysoPC	Lysophosphatidylcholine
mo	Month
OGTT	Oral glucose tolerance test
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PCA	Principal component analysis
ROC	Receiver operating characteristic
SGA	Small for gestational age
SMD	Standardized Mean Difference
DOHaD	The developmental origins of health and disease
y	Year

1. Introduction

Gestational diabetes mellitus (GDM) is one of the most common pregnancy complications, with a prevalence ranging from 1% to > 30% worldwide [1,2] and an incidence of 14.8% in China [3]. GDM is associated with maternal hyperglycemia, which is believed to detrimentally contribute to the programming of fetal metabolism and eventually results in short-term manifestations, including large for gestational age (LGA) or macrosomia, and long-term health risks, such as metabolic dysfunction later in life [1,4].

According to the 1000-day concept, environmental factors, mainly nutrient intake, during a short period after birth are vital for the development and long-term health of offspring [5–9]. Given that human breast milk (HBM) provides essential substrates for the optimal growth of breastfed infants [10], both the length of breast feeding (BF) and the composition of HBM may play a critical role in neonatal growth. Indeed, accumulating evidence has shown that infants of women with normal pregnancies who were exclusively breastfed had a lower fat mass and smaller increases in weight and length at 12 month (mo) than formula-fed infants [11] and that the levels of 2-palmitoyl-glycerylphosphorylethanolamine and carnitine in HBM were positively correlated with infant weight or infant adiposity, respectively, at 1 mo of age [12]. Moreover, human milk oligosaccharides (HMOs), like lacto-N-neo-tetraose and 2′-fucosyllactose, were associated with child height and weight during the first 5 years (y) postpartum [13]. In line with this evidence, the World Health Organization (WHO) recommends exclusive BF for the first 6 mo, with BF continuing to be an important part of the diet until the infant is at least two years old [14]. However, changes in maternal circulating metabolites can affect the composition of milk [15], while GDM leads to maternal metabolic disturbance. Therefore, GDM may profoundly impact HBM composition and the effects of breastfeeding. At this point, the effects of GDM on the HBM metabolome and the optimal BF length for GDM offspring remain unknown.

HBM is divided into 3 types according to the period of lactation: colostrum, transitional milk, and mature milk. Colostrum is present from delivery to approximately 5 d postpartum [16]. Transitional milk occurs from 6 d to 15 d postpartum, while mature milk is produced from 15 d postpartum until weaning [17]. Although ample evidence has shown that the metabolome significantly varies among colostrum, transitional milk, and mature milk in normal pregnancies, the variation in differentiating components in these types of HBM during lactation is still unclear. Most importantly, whether GDM HBM demonstrates different metabolomic alterations during lactation and the effects of such discrepancies on neonatal growth have yet to be elucidated.

In this case–control study, colostrum, transitional milk and mature milk were prospectively collected from women with normal and GDM-complicated pregnancies and then subjected to metabolomic profiling by liquid chromatography tandem mass spectrometry (LC-MS/MS). Growth assessment of neonates was carried out at birth and at 10 days (d), 42 d and 1 y of age, and the correlations between differential metabolites in certain types of HBM and neonatal growth trajectory were investigated.

2. Subjects and methods

2.1. Recruitment of subjects

A total of 227 mothers complicated with GDM (n = 98) and normal women with uncomplicated pregnancies (n = 129) admitted to the Department of Obstetrics, the First Affiliated Hospital of Chongqing Medical University (Chongqing, China), from January 2016 to December 2016, met the inclusion criteria and were recruited into this study. GDM was diagnosed according to the International Association of Diabetes and Pregnancy (IADPSG) guidelines as follows: a 75 g oral glucose tolerance test (OGTT) that resulted in fasting glucose ≥5.1 mmol/l, 1-h ≥10 mmol/l, or 2-h ≥8.5 mmol/l. Eligibility criteria included intention to exclusively breastfeed after delivery and willingness to provide expressed breast milk samples. Pregnancies complicated with pregestational diabetes mellitus, preterm or postterm delivery, advanced maternal age (>40 y) and other major pregnancy diseases, such as

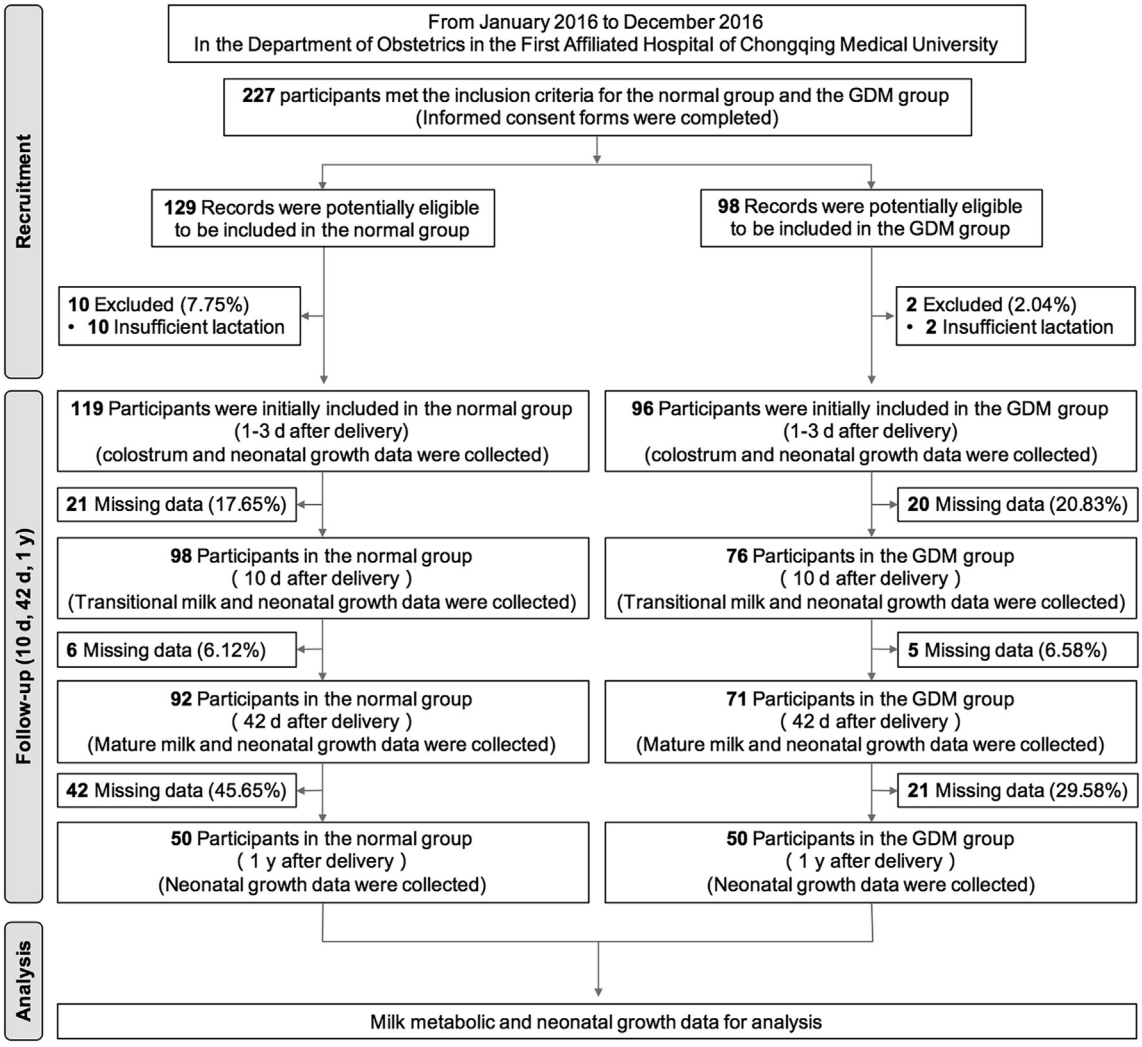


Fig. 1. Flow chart of the study.

preeclampsia, intrahepatic cholestasis, thyroid dysfunction and hepatitis B, were excluded. Due to loss to follow-up (n = 69 in the normal group and n = 46 in the GDM group) or insufficient milk produced by mothers (n = 10 in the normal group and n = 2 in the GDM group), 50 GDM and 50 normal participants who completed neonatal growth assessments and milk sampling at all designated visits qualified for inclusion in the final analyses. The flow chart of the overall study design is shown in Fig. 1.

2.2. Neonatal growth assessments

Neonatal growth assessments were performed at 0 d, 10 d, 42 d and 1 y of age. Infant body weight (BW) and body length (BL) were measured by using a digital measuring bed (Beideneng, Shanghai, China) with accuracies within 0.1 kg and 0.1 cm, respectively. Head circumference was measured with a nonelastic measuring tape.

2.3. Ethics approval

This study was performed in compliance with the Declaration of Helsinki. All procedures were approved by the ethics committee of the First Affiliated Hospital of Chongqing Medical University (No. 2016-65). Written informed consent was obtained from all participants.

2.4. Breast milk sampling

The breast milk samples were obtained at 1–3 d (colostrum), 10 d (transitional milk), and 42 d (mature milk) postpartum. In the morning, whole milk from each mother was collected simultaneously and completely from both breasts by pump expression before feeding the newborn in the hospital. A total volume of 3 mL (colostrum) or 10 mL (transitional or mature milk) was collected. Then, all samples were snap-frozen, stored in liquid nitrogen and transported to the laboratory. The whole milk was homogenized by vortexing the tube for 30 s, and a total of 2 mL (colostrum) or 6 mL (transitional or mature milk) of homogenized milk was transferred into new sterile microcentrifuge tubes in aliquots and immediately stored at −80 °C until analysis.

2.5. Sample preparation and metabolic analysis

2.5.1. Metabolite extraction from milk

Milk samples were thawed on ice and vortexed thoroughly. To extract hydrophilic metabolites, 100 μL of the homogeneous milk sample was mixed with 400 μL methanol (prechilled to −80 °C). The mixture was vortexed for 30 s and incubated for 6–8 h at −80 °C. After centrifugation at 12,000 g at 4 °C for 10 min, the supernatant (300 μL) was transferred to a fresh tube and

lyophilized under vacuum. The dried samples were reconstituted in 80 μL 80% methanol, vortexed for 30 s, and incubated at 4 °C for 15 min. The samples were centrifuged at 12,000 g at 4 °C for 20 min. Finally, 20 μL of supernatant was used for LC/MS-MS analysis.

2.5.2. Metabolite profiling analysis

In positive mode, an amide column (BEH amide, 2.1 × 100 mm, 1.7 μm, Waters, USA) was used for separation with a column temperature of 35 °C. BEH indicates that the columns use Bridged Ethylene Hybrid particles. Separation was initiated with 1% mobile phase B and a flow rate of 300 μL/min. The gradient is shown in Supplementary Fig. S1A. In negative mode, a reversed phase column (BEH C18, 2.1 × 100 mm, 1.7 μm, Waters, USA) was utilized. The column temperature was 35 °C. The gradient was initiated at 1% mobile phase B with a flow rate of 250 μL/min. The gradient is shown in Supplementary Fig. S1B. Untargeted metabolite screening was performed on a Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, USA) after calibration following the manufacturer's guidelines (Thermo Fisher Scientific, USA). The detailed mass spectrometer parameters are listed in Supplementary Fig. S1C.

Metabolites were identified and quantified using Trace Finder 3.2 (Thermo Fisher, USA) based on a home-built database [18]. The in-house database contains ~3500 endogenous metabolites which can be applied for MS identification. MS/MS standard spectra of ~1500 metabolites were included for MS/MS confirmation. Two levels of metabolite identification were achieved based on the in-house database, one with MS/MS confirmation and the other with potential assignment according to accurate ion masses. Only identified/potentially assigned metabolites were used for statistical analysis. Ion features without annotation were not included in the analysis. The relative ion intensity was normalized to the sum of the peak area of a sample.

2.5.3. UPLC-MS mobile phases

For untargeted metabolite screening, a Q Exactive orbitrap mass spectrometer was used. In positive mode, mobile phase A was prepared by dissolving 0.63 g of ammonium formate in 50 mL of HPLC-grade water and then adding 950 mL of HPLC-grade acetonitrile and 1 μL of formic acid. Mobile phase B was prepared by dissolving 0.63 g of ammonium formate in 500 mL of HPLC-grade water, followed by 500 mL of HPLC-grade acetonitrile and 1 μL formic acid. In negative mode, mobile phase A was prepared by dissolving 0.3953 g of ammonium bicarbonate in 1 L of HPLC-grade water (pH~8.0). Mobile phase B is HPLC-grade acetonitrile. All mobile phases were freshly prepared to avoid bacterial contamination.

For data quality assessment, quality control (QC) samples were generated by mixing equal aliquots of representative subsets of subjects. Five injections of QC samples were analyzed at the beginning of the sample queue for column conditioning followed by the analysis of one QC sample for every 18 samples. The sequence order of all the samples was randomized to avoid interference from system instability.

2.6. Statistical analysis

For clinical demographic data (i.e., age and body mass index (BMI)) of mothers or infants and neonatal growth data (i.e., BW and BWG), normally distributed variables are summarized as the mean ± standard deviation (SD) values and were analyzed with Student's t test; nonnormally distributed variables are described as medians (interquartile ranges, IQRs) and were analyzed with Mann–Whitney test. Categorical data are described as percentages and were analyzed with Fisher's exact test. For analysis among multiple groups, ordinary one-way ANOVA was used for normally distributed variables, and the Kruskal–Wallis test was used for nonnormally distributed variables (GraphPad Prism 8.0).

For metabolic data, the initial data analyses were conducted using principal component analysis (PCA) for the exploration of the sample distributions (R-based package ggplot2, v3.3.2). For the identification of significantly different metabolites among colostrum, transitional milk and mature milk in the normal and GDM groups, Student's t test was used (GraphPad Prism 8.0), followed by ROC curves to test the sensitivity and specificity of the metabolites with p-values <0.05 (GraphPad Prism 8.0). Areas under the curve (AUCs) with 95% confidence intervals (CIs) are reported. Differences between ROC curves were tested using the Wilson/Brown test. Finally, once the significant metabolites were selected, a Spearman correlation was performed between each selected significant metabolite and fetal growth parameters (BMI, BWG and BL increments) (SPSS 23.0).

All statistical tests were two-sided, and $P<0.05$ indicated statistical significance, unless otherwise stated.

3. Results

3.1. Clinical characteristics of mothers

The demographic and clinical information of the mothers is presented in Table 1. There were no differences in prepregnancy age or prepregnancy BMI between the two groups. Moreover, education, smoking status, parity, gestational weight gain, gestational age and mode of delivery did not differ between the two groups. Nevertheless, compared to women in the normal group, women in the GDM group demonstrated significantly higher blood glucose levels, not only at fasting but also at 1 and 2 h after the OGTT. To verify that the selected samples are representative of the overall population and that there was no potential deviation in baseline due to exclusion of subjects, the demographic and clinical characteristics of all 227 participants initially recruited were also analyzed. Similarly, the only discrepancy between the GDM and normal subjects was the result of the OGTT (Supplementary Table S1), indicating that the chosen sample represents the differences between all GDM and normal participants.

3.2. Metabolome of breast milk from normal and GDM mothers

In total, 300 HBM samples from the GDM and normal groups (colostrum, transitional milk and mature milk) were subjected to nontargeted metabolomic analysis. The PCA results showed significant differences among colostrum, transitional milk and mature milk in either the GDM or normal group, while the same type of HBM demonstrated similarity between the GDM and normal groups. In addition, the stability and repeatability of the samples in each group were verified (Supplementary Fig. S2 A). After peak alignment and missing value removal, 445 electrospray ionization positive-mode (ESI^+) features and 230 electrospray ionization negative-mode (ESI^-) features were detected in all samples (Supplementary Table S2). In total, 620 compounds were identified by the use of a home-built database. Based on further analysis of the identified compounds by using the Human Metabolome Database (HMDB, http://www.hmdb.ca/), 78 compounds including keto acids and derivatives and furans were present in the HBM in addition to 167 carboxylic acids and derivatives, 120 glycerophospholipids, 104 fatty acyls, 98 organic compounds, 41 nucleotides and 12 benzenoids (Supplementary Fig. S2 B).

Table 1
Demographic and clinical characteristics of mothers.

Variables	Total (n = 100)	Normal (n = 50)	GDM (n = 50)	p-value
Mothers				
Prepregnancy age, years[a]	28.00 (5.00)	28.00 (5.25)	29.00 (5.00)	0.2122
Prepregnancy BMI[b]	20.50 ± 2.00	20.44 ± 1.78	20.57 ± 2.21	0.7419
Education[c]				0.7858
≤Senior high school	16 (16.00%)	9 (18.00%)	7 (14.00%)	
>Senior high school	84 (84.00%)	41 (82.00%)	43 (86.00%)	
Smoking[c]				>0.9999
No	99 (99.00%)	49 (98.00%)	50 (100.00%)	
Before pregnancy only	1 (1.00%)	1 (2.00%)	0 (0.00%)	
Parity[c]				0.3262
0	79 (79.00%)	42 (84.00%)	37 (74.00%)	
≥1	21 (21.00%)	8 (16.00%)	13 (26.00%)	
Gestational weight gain, kg[b]	15.45 ± 4.66	16.09 ± 4.64	14.82 ± 4.64	0.1737
OGTT, mmol/L				
Fasting[a]	4.70 (0.50)	4.55 (0.40)	4.85 (0.63)	0.0001
1-h[b]	8.70 ± 1.83	7.54 ± 1.27	9.85 ± 1.56	<0.0001
2-h[a]	7.65 (2.45)	6.60 (1.43)	8.95 (1.45)	<0.0001
Gestational age, weeks[b]	39.48 ± 0.96	39.52 ± 1.05	39.44 ± 0.87	0.6805
Cesarean delivery[c]	57 (57.0%)	29 (58.0%)	28 (56.0%)	>0.9999

OGTT, oral glucose tolerance test.
[a] Data are the median (IQR), and the Mann–Whitney test was used to compare the normal and GDM groups.
[b] Data are the mean ± SD, and Student's t test was used to compare the normal and GDM groups.
[c] Data are n (%), Fisher's exact test was used to compare the normal and GDM groups.

3.3. Metabolomic changes in HBM from normal and GDM mothers

As shown above, the composition of HBM not only varies at different periods but also differs between GDM and normal women. To explore whether the differences in HBM might be caused by GDM, the expression patterns of metabolites from colostrum to mature milk in the GDM and normal groups were identified. The fold change (FC) calculated by dividing the abundance in transitional milk into the abundance in colostrum was converted to Log2 (FC) and designated Log2 (FC1). Likewise, Log2 (FC2) represented the changes in abundance in mature milk compared to that in transitional milk. Therefore, the 620 detected compounds were categorized into 9 patterns of change (colostrum-transitional milk, transitional milk-mature milk): pattern 1 (unchanged–unchanged), pattern 2 (unchanged-increased), pattern 3 (unchanged-decreased), pattern 4 (increased-unchanged), pattern 5 (increased -increased), pattern 6 (increased-decreased), pattern 7 (decreased-unchanged), pattern 8 (decreased-increased), and pattern 9 (decreased–decreased) (Supplementary Table S3). The top 5 differentially regulated HBM metabolites in each pattern are depicted in Fig. 2A.

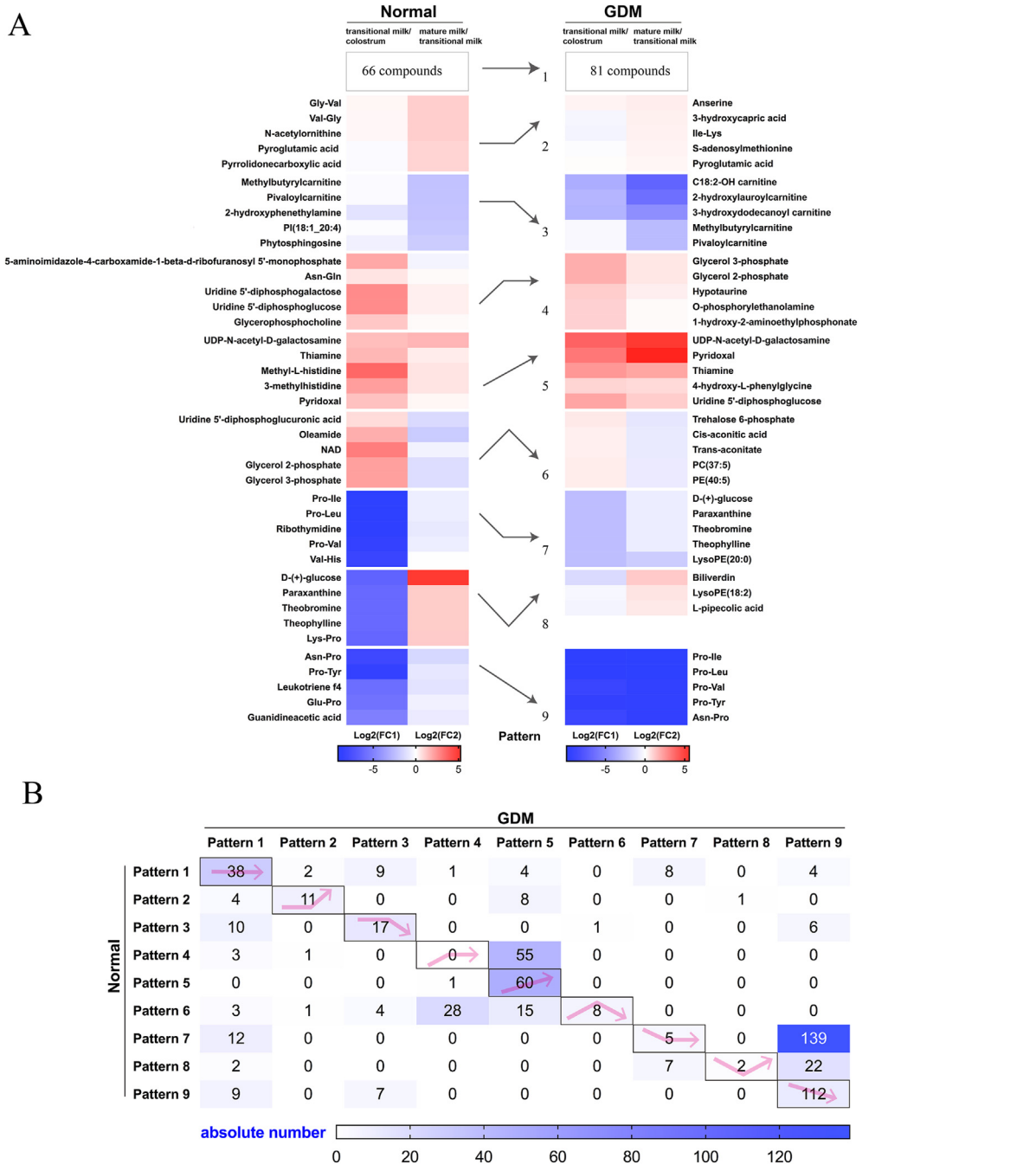
There are similarities in metabolites in individuals with the same variation pattern in the normal and GDM groups. For instance, pyroglutamic acid is in pattern 2 of both normal and GDM women, while methylbutyrylcarnitine, pivaloylcarnitine and 2-hydroxyphenethylamine are all categorized into pattern 3 in both groups; UDP-N-acetyl-D-galactosamine, thiamine and pyridoxal were shown in pattern 5, and Asn–Pro and Pro–Tyr were associated with pattern 9 in the two groups. Nevertheless, metabolites demonstrating notable changes in variation patterns have also been found in GDM breast milk. For example, D-(+)-glucose, paraxanthine, theobromine and theophylline changed to pattern 7 from pattern 8 in the normal group, whereas glycerol 2-phosphate and glycerol 3-phosphate switched from pattern 6 in the normal group to pattern 4 in the GDM group.

We next investigated the predominant pattern shifts of the metabolites with different patterns between the normal and GDM groups. As shown in Fig. 2B and Supplementary Table S2, the variation patterns of 253 metabolites (in the black box), which account for nearly 40% of the total identified metabolites in HBM during lactation, including 49 carboxylic acids and derivatives (mainly nonessential amino acids (AAs)), 17 nucleotides, 48 saturated lipids and 70 unsaturated lipids, were the same between the GDM and normal groups, suggesting that these are fundamental components of HBM that are not affected by GDM.

On the other hand, the metabolites exhibiting different variation patterns between the two groups during lactation could be the components impacted by GDM. Among them, the metabolites demonstrated the opposite tendency of alteration during any two consecutive phases between the GDM and control groups, namely, the switch between increase and decrease at either stage, colostrum-to-transitional milk or transitional milk-to-mature milk, was supposed to be significant. Therefore, we focused on the metabolites that showed variation pattern transitions between patterns 2 and 3, 2 and 6, 2 and 9, 5 and 6, 6 and 8, and 8 and 9. In total, 38 metabolites were found. In detail, only 1 metabolite, oleamide, switched from pattern 6 in the normal group to pattern 2 in the GDM group, while 15 compounds, including 7 lipids, 3 nucleotides and 5 organic acids, switched from pattern 6 in the normal group to pattern 5 in the GDM group. Moreover, 22 compounds, including 14 AAs and 3 sugars, were categorized into pattern 8 in the normal group and shifted to pattern 9 in the GDM group. Intriguingly, these transition of variation patterns all occurred from transitional milk to mature milk. Moreover, the metabolites exhibiting different patterns in the two groups were the featured components of HBM at certain lactation stages.

3.4. Differences in metabolites between the GDM and normal groups

Furthermore, we investigated the 79 discriminating metabolites between GDM and normal HBM in each stage of lactation (Fig. 3 and Supplementary Table S4). In colostrum, 7 metabolites (2 lipids, 2 AAs and 3 organic acids) were found to be decreased and 5 metabolites (1 lipid, 2 AAs, 1 nucleotide and 1 organic acid) were found to be increased in the GDM group compared to the levels in the normal group, whereas 30 metabolites, including 22 glycerophospholipids and 2 fatty acids, were lower and 19 compounds, including 6 AAs, 3 fatty acids and 3 nucleotides, were higher in the transitional milk of the GDM group than in that of the normal group. For mature milk, 10 metabolites, including 3 lipids and 6



Normal \ GDM	Pattern 1	Pattern 2	Pattern 3	Pattern 4	Pattern 5	Pattern 6	Pattern 7	Pattern 8	Pattern 9
Pattern 1	38	2	9	1	4	0	8	0	4
Pattern 2	4	11	0	0	8	0	0	1	0
Pattern 3	10	0	17	0	0	1	0	0	6
Pattern 4	3	1	0	0	55	0	0	0	0
Pattern 5	0	0	0	1	60	0	0	0	0
Pattern 6	3	1	4	28	15	8	0	0	0
Pattern 7	12	0	0	0	0	0	5	0	139
Pattern 8	2	0	0	0	0	0	7	2	22
Pattern 9	9	0	7	0	0	0	0	0	112

Fig. 2. Variation patterns shift of metabolites in GDM and normal breast milk. (A) Metabolites ranking in the top 5 in terms of monolithic variation (|Log2 (FC1)|+|Log2 (FC2)|) of each pattern. FC1 represents the ratio of the compound's expression in transitional milk to its expression in colostrum, and FC2 represents the ratio of the compound's expression in mature milk to its expression in transitional milk. When the P-value was ≥ 0.05 (unpaired Student's t test), the trend was considered unchanged. When the P-value was < 0.05, the trend was considered to increase when Log2 (FC) was > 0 and decrease when Log2 (FC) was < 0. The variation patterns are graphically represented by arrowed lines in the middle. The numbers of compounds categorized into pattern 1 in the normal and GDM groups are provided. (B) Pattern shifts of metabolites between the GDM and normal groups. The black-bordered rectangular grids with pink variation pattern symbols represent the number of metabolites showing the same pattern in the two groups.

organic acids, were downregulated in the GDM group, while 18 metabolites, mainly 6 lipids, 7 organic acids, 3 nucleotides and 2 vitamins, were elevated. Among these metabolites, 4-pyridoxic was the only differentiating metabolite and was upregulated in HBM at all stages in the GDM group compared to the levels in the normal group, while L-pipecolic acid and malic acid were decreased in both colostrum and mature milk in the GDM group. Moreover, 42 of the 79 metabolites were discordant only in transitional milk, while 19 metabolites were found to vary only in mature.

In addition, 5 metabolites—pantothenic acid, riboflavin, pyridoxal, 4-hydroxy-L-phenylglycine and O-succinyl-L-homoserine—were increased in both transitional milk and mature milk in the GDM group compared with the levels in the normal group. However, only LysoPC (18:2) showed lower expression in transitional milk and higher expression in mature milk in the GDM group. These results revealed that GDM is mainly associated with lipids, AAs, and energy-providing nutrients in the metabolome of HBM.

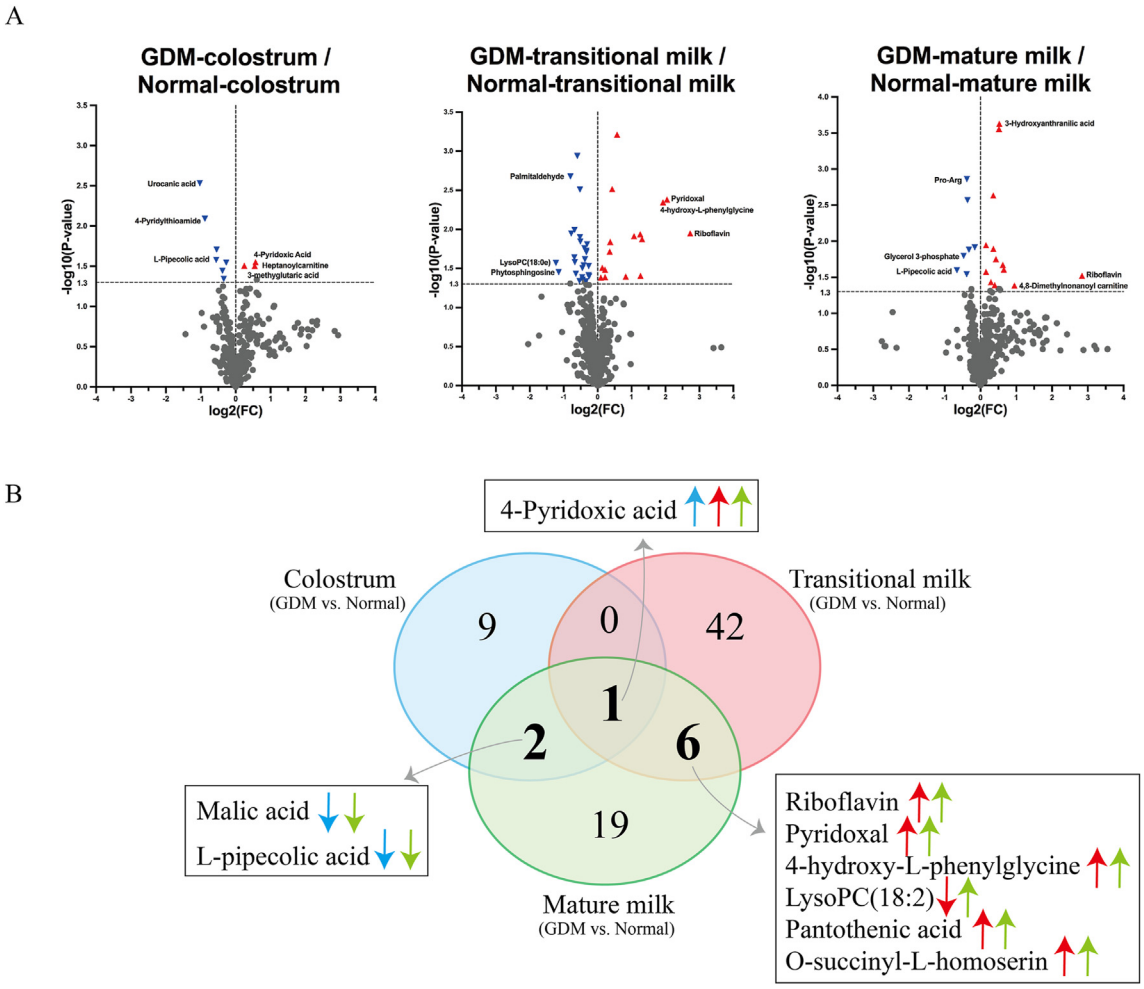


Fig. 3. Metabolites with differences in colostrum, transitional milk and mature milk between the GDM and normal groups. (A) Volcano plot of metabolites in the GDM and normal groups comparing the fold changes and p-values of individual metabolites. Vertical dashed lines indicate a onefold change, and horizontal lines indicate the p-value cutoff of 0.05. The upper right and upper left quadrants contained significant compounds upregulated (red triangle) or downregulated (blue triangle) in comparison to the levels in the normal group with a p-value<0.05; (B) Venn diagram showing the overlap of the different metabolites detected in the colostrum, transitional milk and mature milk between the GDM and normal groups. The compounds in the box indicated by the gray arrow correspond to the overlapping parts. The arrows on the right side of the compound name represent the changing trends of the compound in colostrum (blue arrow), transitional milk (red arrow) or mature milk (green arrow) in the GDM group compared to the normal group from left to right. Red arrow: increase; Blue arrow: decrease.

3.5. The impact of breastfeeding duration on the growth of GDM and normal neonates

To ascertain whether GDM HBM influences the growth of neonates, anthropometric assessments of neonates were performed at 0 d, 10 d, 42 d and 1 y of age. First, the GDM and normal neonates demonstrated similar incidences of LGA and abnormal Apgar scores and comparable head circumference (HC) at birth (Supplementary Table S5). The representativeness of the selected subjects of the 227 neonates initially recruited was further confirmed, except for a slight difference in the incidence of LGA (Supplementary Table S6). Then, the growth trajectory of neonates in the first year after birth was plotted, which demonstrated that BMI did not differ between the normal and GDM groups at any of the aforementioned time points, while the BW of GDM offspring was significantly higher at 1 y; BL was significantly greater only in the GDM group on the 10th day after birth (Fig. 4A). For a more specific assessment, the increments in BW, BL and BMI in the phases of 0–10 d, 10–42 d, and 42 d-1 y were determined. The results showed that the only difference between the GDM and normal groups was BWG from 42 d to 1 y (Fig. 4B).

In this study, 12 normal and 9 GDM women breastfed their children for less than 6 mo. Given that the length of BF has been reported to be positively correlated with neonatal BW within two years after birth [19], the growth of neonates was further analyzed by dividing them into groups according to BF for more or less than 6 mo. Intriguingly, the only significant difference was that the GDM infants exhibited higher BW than normal infants at 1 y old when both were breastfed more than 6 mo (Fig. 4C), implying that persistent BF with GDM breast milk might be associated with accelerated weight gain in the first 1 y of life. Furthermore, the effects of BF duration on neonatal growth from 42 d to 1 y were examined. The duration of BF was not associated with BWG in normal neonates during this period. However, independent of the duration of BF, BWG was markedly increased in the GDM infants compared to that in the normal infants in the BF > 6 mo group (Fig. 4D). However, parallel increases in BMI and BL were found in all groups.

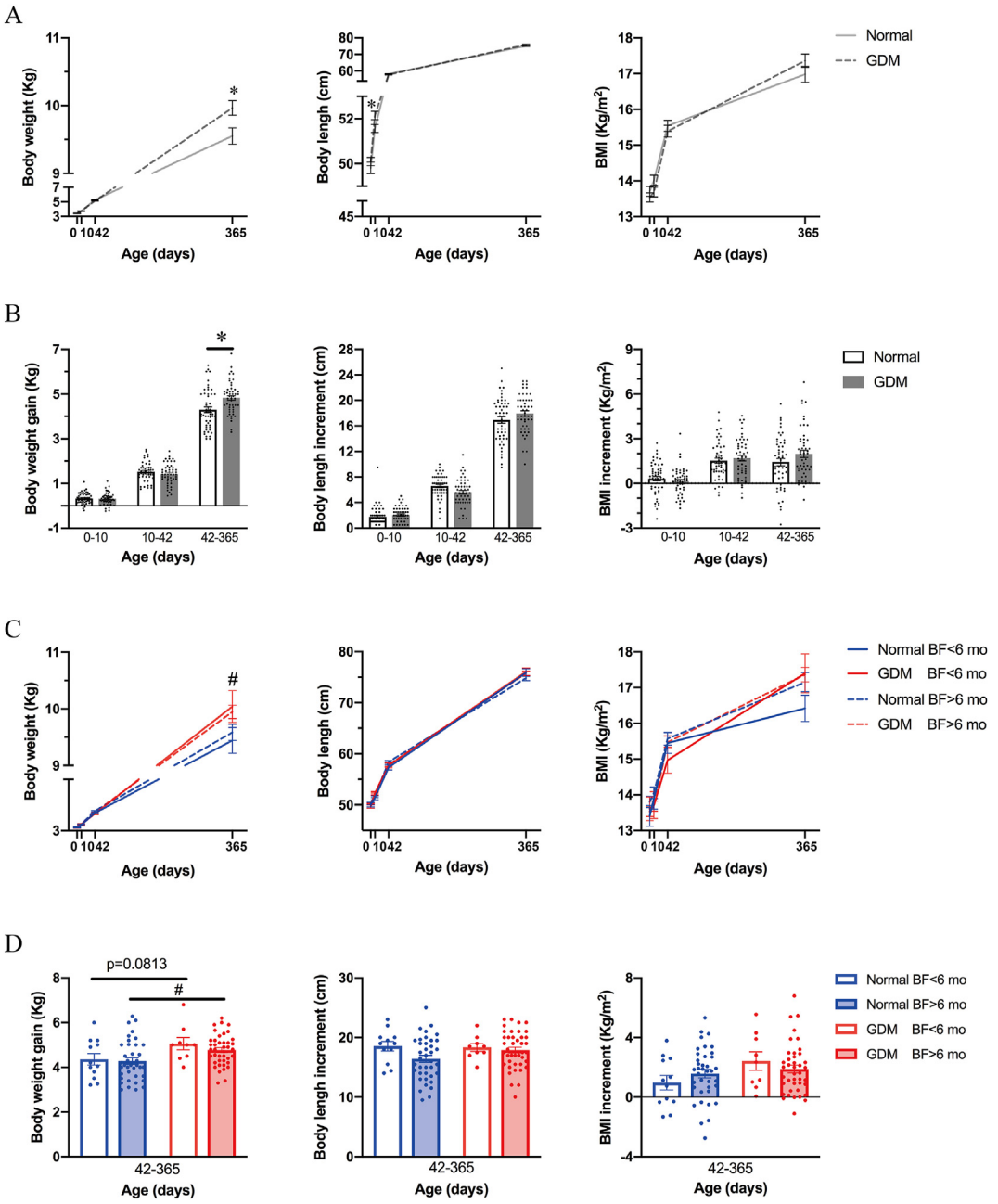


Fig. 4. Neonatal growth curves and BWG, BL increment and BMI increment from delivery to 1 y postpartum. (A, C) Schematic presentation of the neonatal growth curve. (B, D) The BWG and increment of BL and BMI at the different time points of 0–10 d, 10–42 d and 42–365 d. n = 9–41/group. *$P<0.05$ *Normal group vs. GDM group*; #$P<0.05$, *Normal BF* > 6 mo *vs. GDM BF* > 6 mo. BF: Breast feeding.

3.6. Metabolomic fingerprints of mature breast milk of women with GDM

The pattern shift analysis demonstrated that GDM may have a more profound impact on the composition of mature milk than colostrum and transitional milk, and the BWG of children with GDM occurred only in the period of BF with mature milk. To further explore the compounds of HBM that are predominantly associated with GDM status. The 38 metabolites exhibited a transition from transitional milk to mature milk, along with the 28 discriminating metabolites in mature milk between GDM and normal pregnancies, including 3 overlapping metabolites, which were subjected to ROC curve analysis (Supplementary Table S7). Twenty-two of the 63 metabolites displayed a significant false discovery rate (FDR) and AUC ranging from 0.60 to 0.70, and riboflavin had the highest AUC value (Fig. 5).

3.7. Correlations between HBM metabolites and aberrant neonatal weight gain in GDM

To determine whether the metabolites in mature milk contributed to the BWG of children with GDM, Spearman correlation was performed with the aforementioned 63 candidate metabolites and BWG 42 d-1 y after birth (Supplementary Table S8). The unsaturated lipids eicosatrienoic acid (FA 20:3) and LysoPC (20:6) were the only two compounds that positively correlated with BWG. In

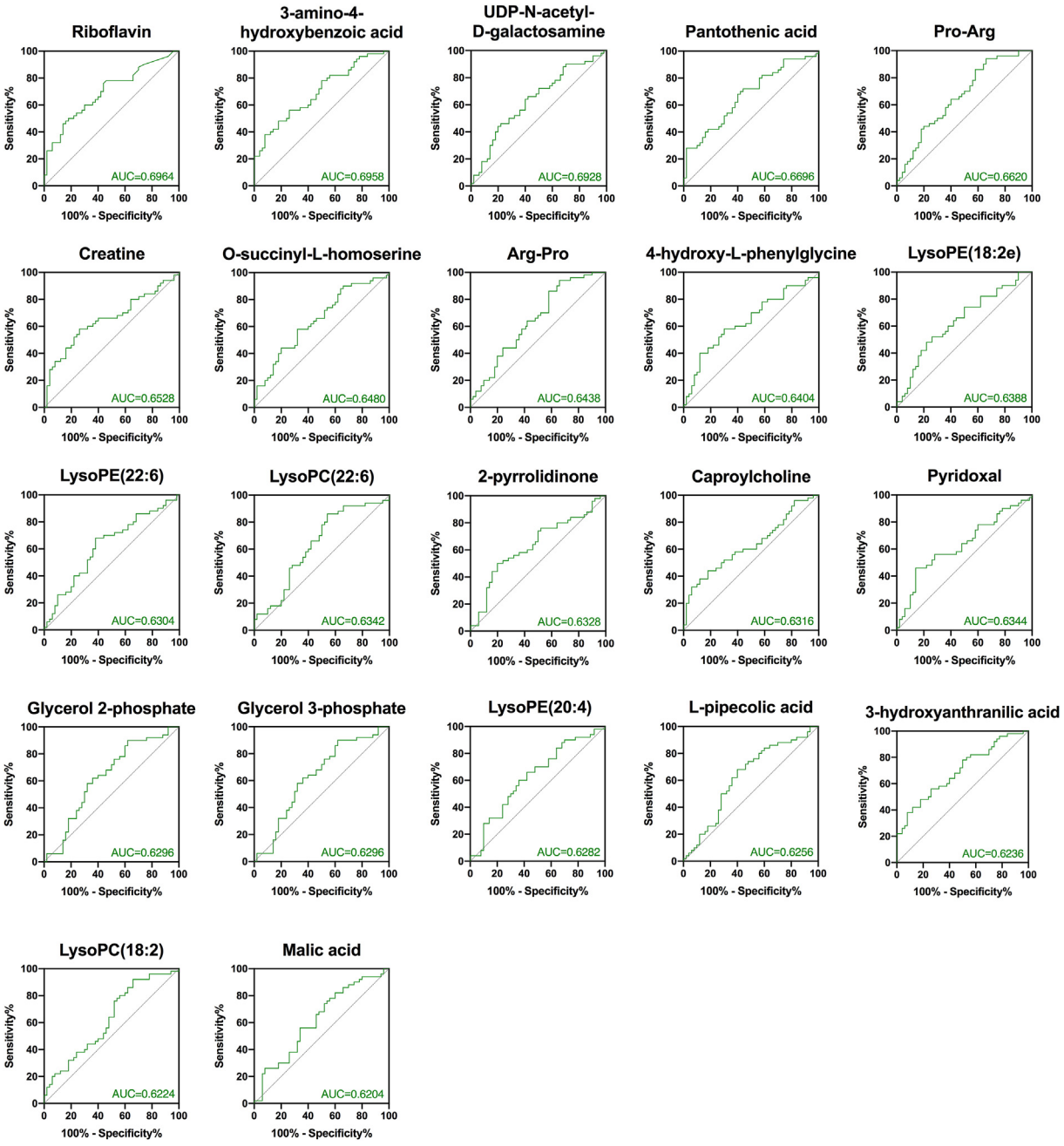


Fig. 5. Receiver operating characteristic (ROC) curve analysis of metabolites in mature milk significantly related to GDM. The performance of each metabolite was evaluated by the areas under the curve (AUCs). Differences between ROC curves were tested using the Wilson/Brown test.

contrast, 10 compounds were negatively correlated with BWG, including phosphocreatine, creatine, D-glutamic acid, N-methyl-D-aspartic acid, L-serine, phosphocholine, iditol, sorbitol, galactitol, and cytarabine (Fig. 6).

4. Discussion

HBM is widely believed to be the best food for newborns, but its composition notably varies among individuals. Thus, the general recommendations for BF need to be tailored according to specific health conditions. As one of the most important endocrine factors postpartum, lactation is closely correlated with the perinatal nutritional status of women. Accumulating evidence suggests that metabolic abnormalities impact the composition of HBM [20]. Although GDM is the most prevalent pregnancy-associated metabolic disease, its impact on the HBM metabolome during lactation has yet to be fully elucidated.

In the present study, colostrum, transitional milk and mature milk samples were prospectively collected from normal and GDM mothers, allowing us to study the effects of GDM on the metabolomic variation patterns throughout lactation, while previous studies investigated the breast milk metabolome of GDM women only at certain stages [2,21–23].

Through the application of LC-MS/MS [24], we identified 620 metabolites in HBM, which is much more than the numbers of metabolites identified in previous reports using gas

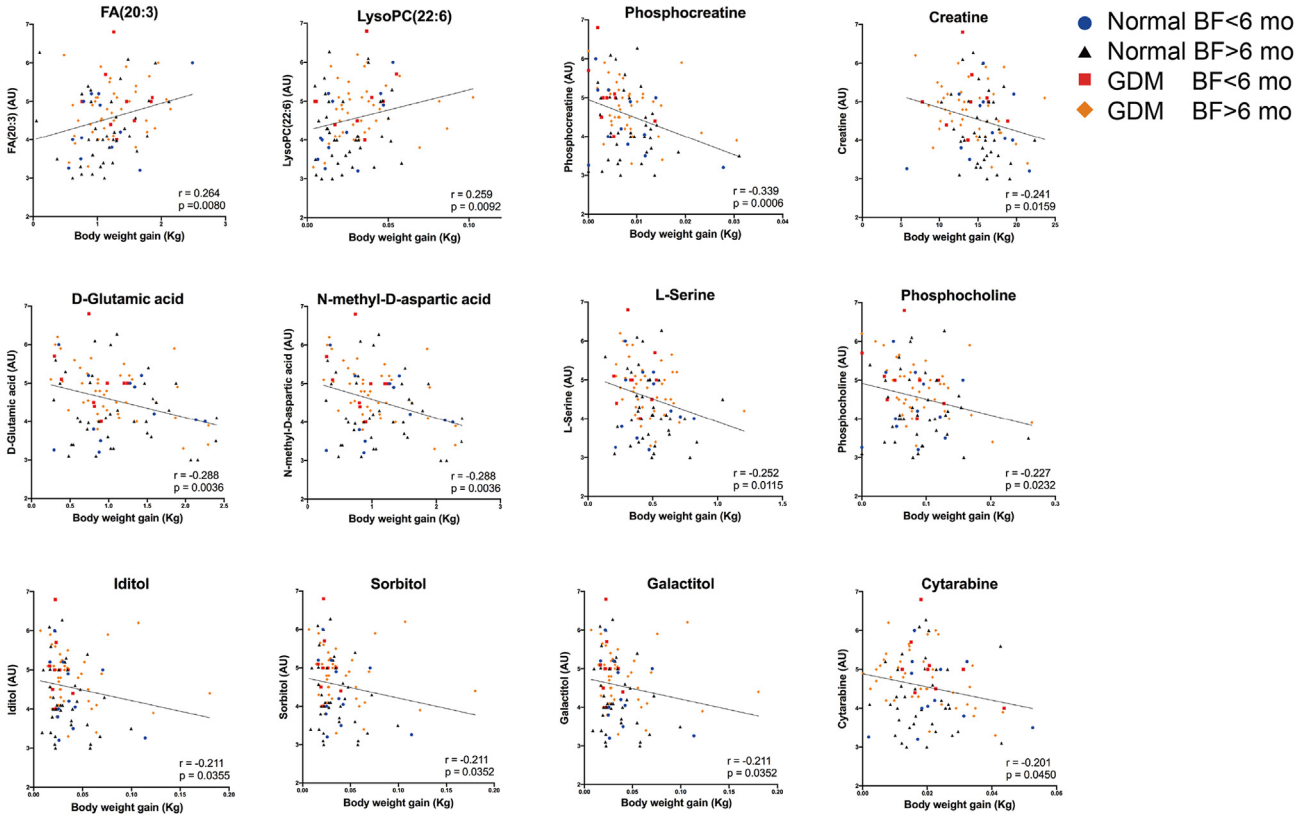


Fig. 6. Spearman analysis of the relationships between metabolites and BWG in mature milk. BF: Breast feeding; AU, arbitrary units.

chromatography-mass spectroscopy (GC-MS) [25] and LC-GC-MS [12]. This finding allowed us to more thoroughly determine the composition of HBM. According to the change in enrichment in different stages of lactation, we categorized the 620 identified metabolites into 9 patterns of variation. The metabolites that remained stable in colostrum, transitional milk and mature milk may compose the basic nutrients of HBM, and the metabolites that showed significant changes in specific stages may be the unique components of the respective type of HBM. Notably, the transition of variation patterns all occurred from transitional milk to mature milk, strongly indicating that GDM may have a more profound impact on the metabolome of mature milk than colostrum and transitional milk.

Although a relatively small sample size was used for the final analysis, the 100 subjects included out of the initial cohort of 227 participants were representative of the original sample. Most importantly, the incidences of LGA in our sample subsets were 12% and 6%, respectively, which is quite close to the reported prevalence of LGA of 13.6% and 7.7% in GDM and non-GDM women with normal weight, respectively, from a large-scale retrospective study that included 9,835 women [26]. Therefore, the selected sample was able to be used to investigate the influence of BF on neonatal growth.

To investigate the potential role of GDM breast milk on neonatal growth trajectory, we carried out a 3-visit pediatric follow-up in the first year after birth, in addition to the anthropologic assessment at birth. Surprisingly, we found that GDM offspring were significantly heavier than control offspring at 1 y due to excessive BWG from 42 d to 1 y. Although a previous study reported that GDM is associated with overweight offspring at the age of 7 y [27], our work adds new evidence that GDM could influence the growth trajectory of offspring at an age as young as 1 y.

Carbohydrates are the most prevalent energy source in HBM; for example, there is an abundance of monosaccharides, sugar alcohols, and oligosaccharides, which account for 40%–50% of the energy of whole milk [28]. We found that three monosaccharides (iditol, galactitol, and sorbitol) were negatively associated with neonatal BWG. Galactitol and sorbitol, the products of galactose and glucose, respectively, are involved in glycolysis to provide energy for infant growth [29]. In addition, HMO can act as a prebiotic, bifidogenic antiadhesive, antimicrobial factor, and immunomodulatory factor [30,31]. In this regard, additional HMOs and monosaccharides in formulas should be recommended for GDM infants.

In addition, lipids are the second most abundant breast milk constituents contributing to infant growth [32]. We found that two unsaturated lipids, eicosatrienoic acid (FA 20:3) and LysoPC (22:6), were positively associated with BWG. Phosphocholine was found to be negatively associated with BWG. Phosphocholine can be converted to phosphatidylcholine to produce LysoPC, which is the main component of oxidatively damaged low-density lipoprotein (oxLDL) [33]. In addition, eicosatrienoic acid (FA 20:3) and LysoPC can be obtained from the diet and can induce the migration of lymphocytes and macrophages, increase the production of proinflammatory cytokines, induce oxidative stress, and promote apoptosis, promoting the development of diseases [34,35]. Furthermore, LPC could promote the uptake of glucose in adipocytes by upregulating the expression of GLUT4, resulting in a reduction in blood glucose in a mouse model of diabetes [36]. Overall, a moderate amount of fat in milk can promote infant growth, but excessive lipids could be associated with excessive neonatal growth.

AAs in HBM are a critical source of protein synthesis building blocks for newborns. In our study, three AAs, N-methyl-D-aspartic acid, L-serine, and D-glutamic acid, were negatively related to BWG.

Although these AAs are traditionally classified as nonessential AAs, they could play important roles in directly regulating key metabolic pathways [37]. In addition, these AAs are related to the TCA cycle and promote ATP production [38] and regulate gene expression, cell signaling, antioxidative responses, and immunity. Moreover, glutamate and aspartate are major sources of metabolic fuel for the small intestine, and they, along with glycine, regulate neurological function [37]. Creatine, which contributes to the transient intracellular storage of metabolic energy and increases muscle mass, and phosphocreatine, the phosphorylated form of creatine, which stores energy for muscle and brain [39], were also negatively associated with BWG. Therefore, we speculate that more of the aforementioned AAs and creatine should be provided in formulas for neonates with GDM.

However, limitations should be noted when interpreting the results. First, we did not detect milk components such as exosomes, hormones and bacteria. Second, parameters such as infant adiposity, total fat mass, total lean mass and central obesity were not assessed in the present study. Third, the correlations between maternal serum and HBM metabolites should be explored in our future studies. Finally, the confounding factor of the degree of severity of impaired glucose tolerance of the mother was not taken into consideration when performing Spearman correlation analysis.

In conclusion, the composition of HBM varies during lactation, and such metabolomic changes are disturbed in GDM. Feeding infants with GDM breast milk leads to excessive BWG from 42 d to 1 y, which might be attributed to the high levels of eicosatrienoic acid (FA 20:3) and lysoPC (22:6) and the deficiency of three monosaccharides (iditol, galactitol, sorbitol) and three AAs (N-methyl-D-aspartic acid, L-serine, D-glutamic acid) in GDM breast milk. Although the effects of these metabolites on children's growth require further clarification, the present study suggests that to prevent excessive growth, breastfeeding length should be carefully tailored for GDM offspring, and infant formula should be modified specifically for GDM offspring to allow them to obtain optimal feeding outcomes.

Author's contributions

The authors' responsibilities were as follows-CT, HQ conceived and designed the study; JY, WW, ZC, JQ, HJ, XL, LW, and JT collected samples and data; XHL performed metabolomics; YW, XYL and XHL analyzed the data; RS, MK and PB interpreted the results; CT, HQ provided funding resources; YW wrote the draft; CT, XHL, MK and PB edited the manuscript. All authors declare no competing interests.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2021.02.014.

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