Article

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Longitudinal Analysis of Urine Metabolomics in Preterm Infants Based on UHPLC-MS/MS Technique

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Background: The metabolic characteristics of preterm infants in the early postnatal period are closely linked to their growth and development. Investigating the corresponding metabolic changes that occur during the early postnatal maturation process of preterm infants may help improve their clinical management. In this prospective cohort study, we aimed to characterize urinary metabolism in preterm infants during the first two weeks of life and explore its relationship with metabolic homeostasis regulation in the early postnatal period.

Methods: Between June 1, 2021, and October 1, 2022, 36 preterm infants eligible for enrollment were recruited from the Children's Hospital of Fudan University (Shanghai, China), and urine samples were collected from these 36 preterm infants on postnatal day 1 (PND1), postnatal day 7 (PND7), and postnatal day 14 (PND14), respectively. A longitudinal urinary metabolomics study of preterm infants was conducted using an Ultra-high performance liquid chromatography-MS/MS (UHPLC-MS/MS) technique to compare urinary metabolic characteristics between PND7 and PND1 during the first postnatal week, and between PND14 and PND7 during the second postnatal week.

Results: Broad-targeted urinary metabolomics analysis of preterm infants during the first week of life identified 75 differentially expressed urinary metabolites, of which 57 were upregulated compared to birth, while 18 were downregulated. The most significantly upregulated metabolite was L-3-hydroxykynurenine ($p = 0.003$), while the most significantly downregulated was N-Acetyltyrosine ($p < 0.001$). The metabolic pathway most significantly enriched among these 75 differentially expressed metabolites was the Arginine biosynthesis ($p = 0.001$). Compared to the first week, only 29 differentially expressed urinary metabolites were identified in preterm infants during the second week after birth, with 22 metabolites upregulated and 7 downregulated. Among them, (3-hydroxyphenyl)-3-hydroxypropionic acid (HPHPA) was the most significantly upregulated ($p = 0.001$), while N-Acetylhistidine was the most significantly downregulated ($p = 0.008$). The metabolic pathways most significantly enriched among these 29 urinary metabolites were Glycolysis/Gluconeogenesis ($p = 0.008$). Cinnamoylglycine, cyclic-3',5'-GMP and lactate were common urinary differentially expressed metabolites in the first and second weeks after birth in preterm infants, and the Glycolysis/Gluconeogenesis pathway was the metabolic pathway significantly associated with urinary differentially expressed metabolites in the first two weeks after birth.

Conclusion: This single-center study describes the general characteristics of urinary metabolomics in preterm infants during the first two weeks after birth. Glycometabolism was identified as a key factor influencing changes in urine metabolism in preterm infants during the first two weeks after birth, providing a reference for metabolic adaptation assessment and clinical management strategies in the early postnatal period.

Clinical Trial Registration: ClinicalTrials.gov (NCT06018831).

Keywords: preterm infants; urine; widely targeted metabolomics; glycometabolism

Introduction

Prematurity has become a global public health concern due to its high incidence and numerous short-term and long-term complications [1]. Approximately 15 million preterm infants are born worldwide each year, and the incidence of preterm birth continues to rise in some countries [2]. Because preterm infants are separated from the intrauterine environment too early, the development of their organ systems and the regulation of key metabolic pathways by various enzymes are not yet fully mature [3]. Given that the extrauterine environment and nutritional supply after birth differ completely from those in utero, the early metabolic characteristics of preterm infants differ from those of healthy term infants [4]. Investigating metabolic changes in preterm infants during the early postnatal period is therefore crucial for improving their clinical management.

Glycometabolic disorders were previously considered the leading cause of metabolic adaptation problems in preterm infants during the early postnatal period [5,6]. However, recent studies have shown that early metabolic changes in preterm infants are also associated with many other factors. For example, preterm birth interrupts the maternal-fetal transfer of essential fatty acids (including docosahexaenoic acid and arachidonic acid) that occurs during late pregnancy, leading to a gradual increase in deficiencies of these nutrients within the first week after birth, which persist for up to one month postpartum; these deficiencies increase the risk of neurodevelopmental impairment in preterm infants [7]. In addition, during the first few weeks of extrauterine life, preterm infants frequently experience insufficient energy accumulation and protein deficiency [8]. Even with aggressive parenteral and/or early enteral feeding, and despite supplementation of calories and protein according to recommended intake levels, this still leads to significant postnatal growth impairment on average [9]. Although these studies provide partial evidence for early metabolic adaptation in preterm infants, they do not fully capture the overall trajectory of this process. New methodological approaches are needed to investigate metabolic adaptation from an intrinsic perspective during the early postnatal period, thereby characterizing the developmental characteristics of preterm infants at this early stage.

Metabolomics, as a molecular phenotype reflecting interactions between genotype and environment, integrates high-throughput, high-resolution analytical techniques with bioinformatics to provide a unique perspective on biological organisms [10,11,12,13]. It is one of the key technological tools in precision medicine [14]. Urine possesses a unique biochemical composition that serves as a functional reflection of the interplay between genotype, physiology, disease, nutrition, and environmental factors and can provide information on an individual's overall metabolic status [15]. Furthermore, urine is not subject to strict internal homeostatic regulation and can accumulate subtle yet sensitive changes related to the whole body; additionally, it can be collected using non-invasive methods [16].

However, the range of metabolites detectable in urine samples is limited, and some metabolites may degrade rapidly over time; therefore, consistency in the timing of sample collection is crucial [17]. Given the current limited understanding of the overall metabolic status of preterm infants, applying metabolomics to urine samples collected during the early postnatal period may provide new insights into the metabolic characteristics of preterm infants from multiple perspectives, including genetic and environmental factors.

This study conducted a longitudinal urinary metabolomics analysis of preterm infants during the first two weeks of life, with urine samples collected at specific postnatal time points: the first day after birth (Postnatal Day 1, PND1), day 7 (PND7), and day 14 (PND14). Using UHPLC-MS/MS, we performed comparative analyses of urine samples collected at these three time points to identify metabolic pathways and molecules closely associated with metabolic adaptation in preterm infants during the first two weeks of life and to explore temporal patterns in their urinary metabolomics profiles.

Methods

Study Population

This prospective, single-center observational cohort study was approved by the Ethics Committee of the Children's Hospital of Fudan University (No. 2020-524). Our study and application materials were fully reviewed, and the committee certified that the study posed no risk to patients and was conducted in accordance with the Declaration of Helsinki. The technical workflow of this study is shown in Fig. 1. We also registered this study on ClinicalTrials.gov; the trial registration number is NCT06018831, https://clinicaltrials.gov/study/NCT06018831. Preterm infants were recruited in the Neonatal Department of the Children's Hospital of Fudan University from June 1, 2021, to October 1, 2022. Guardians of preterm infants willing to participate in the study were informed of the study objectives, as well as the methods, timing, and frequency of sample collection. Guardians who agreed to participate signed an informed consent form and were permitted to withdraw from the study voluntarily at any time. Inclusion criteria for preterm infants included: (1) Gestational age (GA) <37 weeks; (2) Birth weight (BW) ≥1000 g; (3) Apgar score ≥8 within 1 minute of birth; (4) Admission within 24 hours of birth; (5) Hospital stay >14 days; (6) Informed consent provided by the guardian and signed on the day of admission; (7) Complete medical records for the preterm infant. Exclusion criteria for preterm infants included: (1) Infants with congenital malformations; (2) Infants with congenital genetic metabolic disorders or chromosomal abnormalities;

(3) Infants with concurrent infections at birth; (4) Infants who developed infections, systemic complications (such as neonatal respiratory distress syndrome, necrotizing enterocolitis, or intracranial hemorrhage), or died during hospitalization; (5) Infants whose mothers have a history of severe medical or surgical conditions (such as heart disease, hypertension, diabetes, infectious diseases, or cancer); (6) Infants whose mothers had severe complications during pregnancy; (7) Infants whose mothers had a history of medication use during pregnancy due to infections or other factors; (8) Infants whose mothers were exposed to radiation or harmful chemicals during pregnancy.

Clinical Data Collection

The clinical data required for this study were mainly obtained from electronic medical records completed by doctors. These included the gender, gestational age (GA), birth weight (BW), Apgar scores at 1/5/10 minutes after birth, whether they were multiple births, the health status of the mother during pregnancy (diseases, medications, nutrition, and lifestyle, etc.), as well as the feeding methods of the premature infants during hospitalization, the postmenstrual age (PMA) and weight of the premature infants on the day of each urine sample collection.

Urine Sample Collection

Urine samples were collected by attaching a sterile urine collection bag (Changzhou Xiaochun Medical Equipment Co., Ltd, Changzhou, China) to the perineum of the preterm infants. The first urine collection was performed on the day of admission (PND1), followed by two additional collections in the morning at 1 week postnatal (PND7) and 2 weeks postnatal (PND14). We collected urine samples from 8:00 AM to 10:00 AM. Time since last feeding was not controlled because preterm infants were fed on demand. Preterm infants with contaminated urine samples and those whose urine samples could not be collected during this time window were excluded from the subsequent study. The dates of the three urine collections and other parameters, such as body weight on the day of collection, were recorded for each enrolled preterm infant. Immediately after collection, urine samples were stored in liquid nitrogen and transported to the laboratory, where they were promptly stored in a –80 °C freezer (Thermo Fisher Scientific, Waltham, MA, USA).

Sample Preparation

Urine samples were obtained from the –80 °C freezer, thawed in an ice bath, and centrifuged at 3000 rpm for 5 minutes at 4 °C. Then, 20 µL of supernatant from the centrifuged urine sample was transferred to a 360 µL 96-well plate, followed by the addition of 80 µL of internal standard extraction solution (internal standard: 4-chlorophenylalanine (TCI Shanghai Development Co., Ltd, Shanghai, China)). The mixture was vortexed for 30 seconds and then incubated at –20 °C for 30 minutes to precipitate trace proteins and inorganic salts. After incubation, the mixture was centrifuged at 4000 rpm for 20 minutes at 4 °C. The supernatant was retained and transferred to a 100 µL sample well for subsequent analysis. Equal volumes of urine samples from preterm infants were mixed and processed using the same procedure to prepare quality control (QC) samples. During instrumental analysis, one QC sample was inserted after every 10 study samples to assess the reproducibility of the entire analytical process. Blank samples consisted of 20 µL of ultrapure water mixed with the internal standard extraction solution and were prepared using the same procedure described above.

LC-MS Collection of Urine Samples

Reverse-phase chromatography was performed using an Acquity UPLC HSS T3 column (1.8 µm, 2.1 × 100 mm). Mobile phase A consisted of H_2O containing 0.1% formic acid (FA, Sigma-Aldrich, St. Louis, MO, USA; Lot: F112037), and mobile phase B consisted of an acetonitrile (ACN, Sigma-Aldrich, St. Louis, MO, USA; Lot: STBL1113) solution containing 0.1% formic acid. The flow rate was set to 0.4 mL/min, and the column temperature was maintained at 40 °C. Prior to each injection, the initial mobile phase gradient was maintained for 5 minutes; the injector temperature was 10 °C, and the injection volume was 1 µL. For hydrophilic chromatography, an Acquity UPLC BEHA Midecolumn (1.7 µm, 2.1 × 100 mm) column was used. Mobile phase A consisted of acetonitrile and water in a 5:95 ratio, containing 0.3% formic acid and 10 mM ammonium formate. Mobile phase B consisted of acetonitrile and water in a 95:5 ratio, containing 0.3% formic acid and 10 mM ammonium formate (Sigma-Aldrich, St. Louis, MO, USA; Lot: 0000521380). The flow rate was set to 0.6 mL/min, and the column temperature was maintained at 45 °C. Prior to each injection, the initial mobile phase gradient was maintained for 5 minutes. The injector temperature was 10 °C, and the injection volume was 1 µL per injection. The mobile phase gradient profile was as follows: 100% mobile phase B was maintained for 1 min, followed by a linear decrease from 100% B to 50% B between 1 and 12 min. All prepared urine samples were randomly numbered using Excel, and the order of injection was determined based on these numbers. This randomized sampling process minimized potential interference from intergroup differences. Urine samples were analyzed using the POS and NEG modes on the HSS T3 column and the POS mode on the BEH Amide column. 10 QC samples were first collected to stabilize the instrument, followed by the analysis of 9 urine samples, with one QC sample collected intermittently after every 9 urine samples.

Identification and Analysis of Metabolic Data

By collecting LC-MS data from urine samples of 36 preterm infants on PND1, PND7, and PND14, we obtained

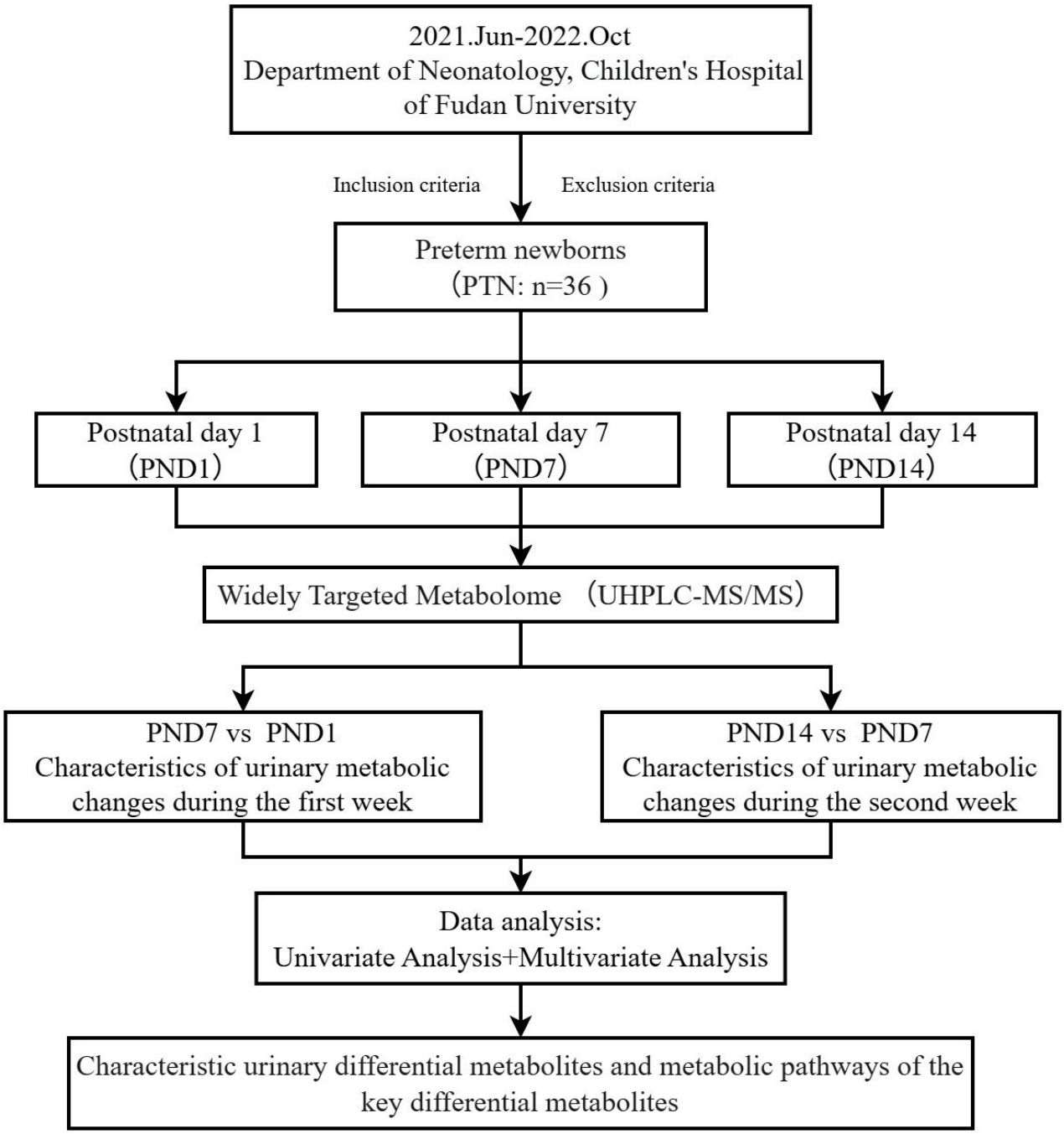

Fig. 1. Technical workflow of this study.

three sets of raw spectra: POS and NEG modes using the HSS T3 column, and POS mode using the BEH Amide column. The raw spectra were converted to. mzXML format using ProteoWizard software 3.0 (https://proteowizard.sourceforge.io/), followed by data normalization and post-processing using R software 3.6.3 (https://www.r-project.org), including peak identification, peak alignment, and extraction of ion peak areas. Missing values were removed using the 80% rule, retaining variables with non-zero values in at least 80% of the samples. Missing values were imputed using the 1/2 minimum value principle. Urine sample mass spectrometry peak response intensities were normalized using the creatinine correction method, while within-batch correction was performed using SVR and internal standard correction methods. Principal Component Analysis (PCA) and Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA) were conducted, with the VIP value used to assess the value of individual variables. This study employed a multidimensional approach combined with the following criteria for identifying differ-

entially expressed metabolites: VIP >1, $p < 0.05$, and FC >1.5 or FC <0.67. False Discovery Rate (FDR) correction was applied to adjust the obtained p-values. Differential metabolites identified in both the cation and anion modes were consolidated, and MetaboAnalyst 5.0 (https://www.metaboanalyst.ca) was used to perform metabolic pathway enrichment analysis. Pathways with a high degree of association were selected using a threshold of $p < 0.05$.

Statistical Analysis

Descriptive statistics were used to characterize the study subjects. A t-test was applied to normally distributed quantitative data, while the Mann-Whitney U test was used for data with a skewed distribution. The Shapiro-Wilk test was used to test for normality, and the Levene test was used to test for homogeneity of variances. Continuous variables, such as gestational age, birth weight, and maternal age at delivery, were expressed as mean ± standard deviation ($\bar{x} \pm s$); Categorical variables, such as gender, 1-minute Apgar score, maternal parity and gravidity, and mode of delivery, were presented as the number of cases (N) and percentages (%). All statistical analyses were performed using IBM SPSS Statistics 23 (https://www.ibm.com/products/spss-statistics). Two-tailed tests of significance were conducted, and $p < 0.05$ was considered statistically significant.

Results

Clinical Characteristics of Enrolled Preterm Infants

In this study, there were 36 preterm infants who met the inclusion criteria and successfully provided 3 urine samples within 2 weeks after birth. The clinical characteristics of these 36 preterm infants were analyzed (Table 1). Among them, 20 were male (55.56%) and 16 were female (44.44%), with an average GA of 33.09 ± 1.38 weeks and an average BW of 2098.69 ± 361.69 g (male: 2111.27 ± 335.71 g; female: 2078.93 ± 411.66 g). The Apgar scores at 1 minute after birth were analyzed, with 15 having a score of 9 (41.67%), 11 having a score of 8 (30.55%), and 10 having a score of 10 (27.78%). The mean maternal age at delivery for the 36 preterm infants was 31.92 ± 4.88 years. Among them, 15 had vaginal delivery (VD) and 21 had cesarean section (CS). Fig. 2 shows the PMA corresponding to the 3 urine samples collected from these 36 preterm infants. Each column of 3 points from bottom to top represents the PMA of 1 premature infant at PND1, PND7, and PND14. Based on the relationship between GA and BW, 3 of these 36 preterm infants were small for gestational age (SGA), with a birth weight below the 10th percentile; the remaining 33 were appropriate for gestational age (AGA), with a birth weight between the 10th and 90th percentiles, indicating good intrauterine development.

Table 1. Clinical characteristics of enrolled preterm infants.

Characteristics	Statistic
Gender	
Female	16 (44.44%)
Male	20 (55.56%)
GA (weeks)	33.09 ± 1.38
BW (g)	2098.69 ± 361.69
BW for males	2111.27 ± 335.71
BW for females	2078.93 ± 411.66
Apgar at 1 min	
8	11 (30.55%)
9	15 (41.67%)
10	10 (27.78%)
Maternal gravidity	
1	10 (27.78%)
2	12 (33.33%)
≥3	14 (38.89%)
Maternal parity	
0	15 (41.67%)
1	18 (50.00%)
≥2	3 (8.33%)
Delivery mode	
VD	15 (41.67%)
CS	21 (58.33%)
Maternal age	31.92 ± 4.88

GA, gestational age; BW, birth weight; VD, vaginal delivery; CS, cesarean section.

Metabolomic Characteristics of Urine in Preterm Infants During the First Week of Life

Urine samples collected from preterm infants on PND1 represent their initial postnatal urinary metabolic status, while those collected on PND7 represent their urinary metabolic status at 1 week of age. By comparing PND7 with PND1, we analyzed changes in urinary metabolism in preterm infants during the first week of life.

Unsupervised PCA revealed partial overlap in the metabolites of urine samples collected on PND7 and PND1. However, when an OPLS-DA model was applied, a trend toward separation between the two groups emerged (Fig. 3A). Based on the screening criteria for differentially expressed metabolites, 75 differentially expressed metabolites were identified in the comparison of PND7 vs. PND1 in preterm infants (shown in **Supplementary Table 1**). At PND7, 57 metabolites were upregulated and 18 were downregulated in the urine of preterm infants (Fig. 3B). Among them, L-3-hydroxykynurenine (L-3-HK, $p = 0.003$), cinnamoylglycine ($p = 0.002$), Galactose 1-phosphate ($p = 0.008$), Mannose-6-phosphate ($p = 0.001$), and Glucose-6-Phosphate ($p = 0.001$) were significantly increased, while N-Acetyltyrosine ($p < 0.001$), 2-Deoxyribose-5-phosphate ($p = 0.002$), Proline betaine ($p = 0.011$), N-Acetyphenylalanine ($p = 0.012$), and 2-Hydroxyadenine ($p = 0.010$) were significantly decreased. Metabolic path-

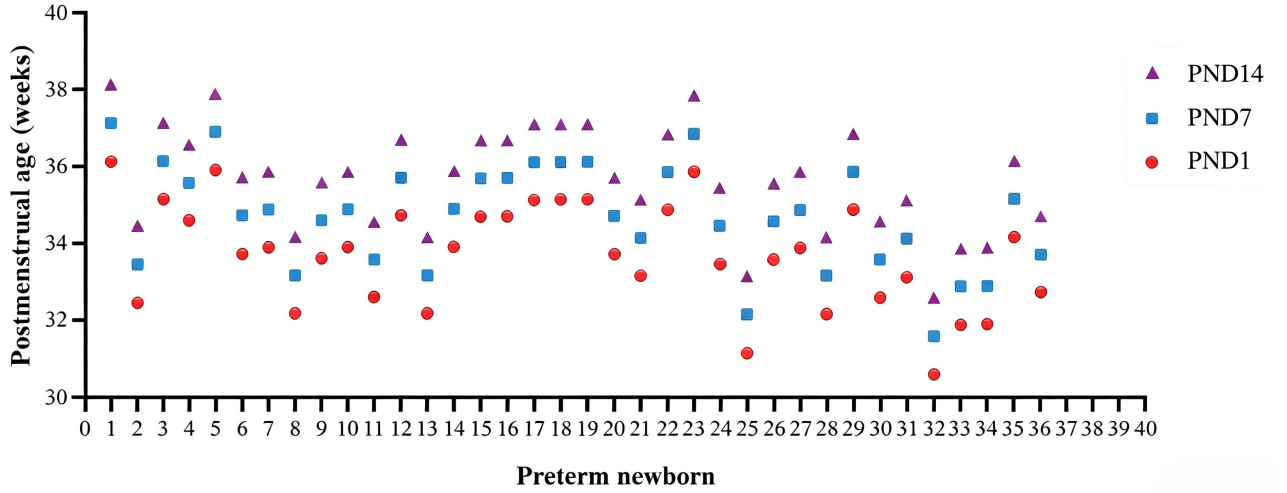

Fig. 2. Scatter plot of PMA distribution at urine collection time in 36 preterm newborns. PMA, postmenstrual age; PND, postnatal day.

way enrichment analysis of the 75 differentially expressed metabolites revealed that the Pentose phosphate pathway (p = 0.002), Arginine biosynthesis (p = 0.001), D-glutamine and D-glutamate metabolism (p = 0.002), Glycolysis/Gluconeogenesis (p = 0.004), Fructose and mannose metabolism (p = 0.025), and Glycine/serine/threonine metabolism (p = 0.045) were closely associated with urinary metabolites in preterm infants during the first week of life (Fig. 3C).

Metabolomic Characteristics of Urine in Preterm Infants During the Second Week of Life

Using urine samples from PND14 as a representative of the metabolic state of preterm infants in the second week after birth, we analyzed changes in urinary metabolism during this period by comparing the metabolic profiles on PND14 and PND7.

Since PCA did not clearly distinguish between the PND14 and PND7 urine samples, an OPLS-DA model was further applied, which revealed differences between the two groups (Fig. 4A). Based on the screening criteria for differentially expressed metabolites, 29 differentially expressed metabolites were identified in the PND14 vs. PND7 comparison (shown in **Supplementary Table 2**). In the second week after birth, 22 metabolites were upregulated and 7 were downregulated in the urine of preterm infants (Fig. 4B).

Among them, 3-(3-hydroxyphenyl)3-hydroxypropionic acid (HPHPA) (p = 0.001), Fructose-1,6-bisphosphate (p = 0.003), and cyclic-3′,5′-GMP (p = 0.004) were significantly upregulated; whereas N-Acetylhistidine (p = 0.008), Agmatine (p = 0.010), and 7-Methylguanine were significantly downregulated (p = 0.012). Glycolysis/Gluconeogenesis (p = 0.008) was significantly associated with these 29 differentially expressed metabolites (Fig. 4C).

A Combined Analysis of Urinary Metabolomic Profiles in Preterm Infants During the First and Second Weeks of Life

To investigate whether there are any associations or patterns in the metabolic adaptation of preterm infants during the first two weeks of life, we conducted a combined analysis of the differences in urinary metabolomics between the first and second weeks. The number of differentially expressed metabolites indicated that urinary metabolic changes in preterm infants were more pronounced during the first week than during the second week (Fig. 5A). Furthermore, metabolic changes in both weeks were predominantly characterized by an increased number of upregulated metabolites, suggesting that the levels of most urinary metabolites detected in this study tended to rise in preterm infants after birth.

Venn diagram analysis of differential metabolites in urine samples from the first and second weeks revealed 3 overlapping differential metabolites (Fig. 5B), namely cinnamoylglycine, cyclic-3, 5′-GMP (cGMP) and lactate. A comparison of the urinary levels of these three metabolites in preterm infants at PND1, PND7, and PND14 was performed (Fig. 5C): cinnamoylglycine showed a gradual increase from birth; lactate was elevated at birth, gradually decreased during the first week, and then slightly rebounded by the second week; cGMP exhibited a trend similar to lactate, but its fluctuations were significantly more stable than those of lactate. Correlation analysis between these three metabolites and the mass spectrometric responses of urinary metabolites at PND1, PND7, and PND14 was performed (Fig. 5D). The metabolic response of lactate was highest on the day of birth, whereas cinnamoylglycine showed the

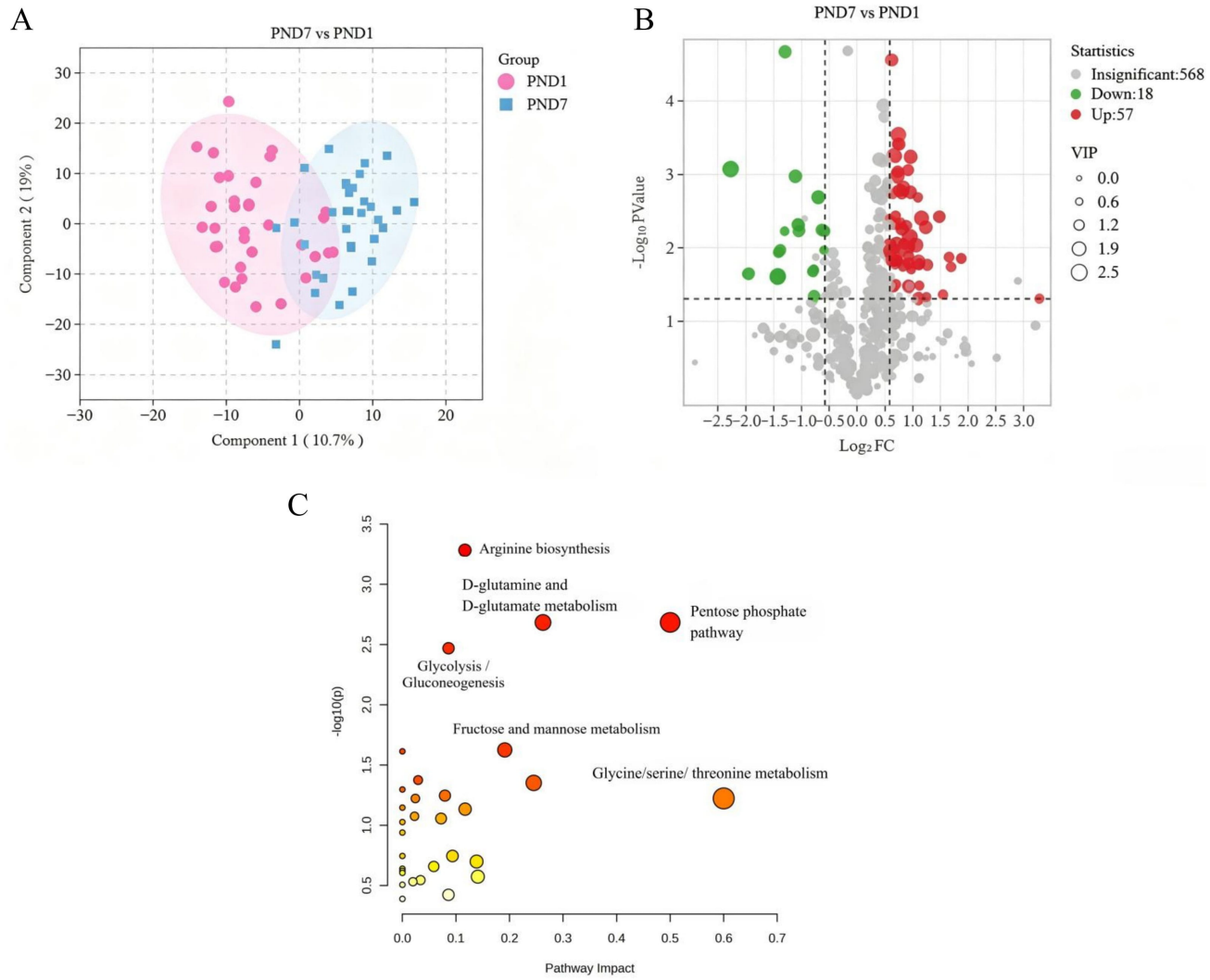

Fig. 3. Analysis of urinary metabolism and enrichment pathways in the first week. Metabolic differences in urine samples from preterm infants between PND7 and PND1: (A) OPLS-DA model for PND7 and PND1urine samples ($R^2X = 0.297$, $R^2Y = 0.629$, $Q^2 = 0.451$); (B) Volcano plot of differentially expressed metabolites in PND7 and PND1 urine samples; (C) Enrichment pathway analysis of differentially expressed metabolites in PND7 and PND1 urine samples. OPLS-DA, Orthogonal Partial Least Squares-Discriminant Analysis; PND, postnatal day.

strongest response during the second week after birth. In contrast, the metabolic response of cGMP remained relatively stable throughout the study period. These findings indicate that these three substances are closely associated with urinary metabolic changes during the first two weeks after preterm birth, with each metabolite exhibiting distinct characteristics.

From the perspective of significantly enriched pathways, a combined analysis was conducted on the urinary metabolic characteristics of preterm infants during the first and second weeks after birth. Glycometabolism was found to be most closely associated with the urinary metabolites of preterm infants during the first two weeks after birth. Among the differential metabolites, they were involved in the Pentose phosphate pathway (PPP), Glycolysis/Gluconeogenesis, Fructose and mannose metabolism and some of these metabolites were mutually transformed and regulated in these pathways (Fig. 6A). In the urinary changes observed in preterm infants during the first week, levels of glucose, Glucose-6-phosphate, Glyceraldehyde-3-phosphate, Glyceric acid-3-phosphate and Ribulose-5-phosphate increased (Fig. 6B), while lactate levels decreased (Fig. 6B), which may indicate enhanced Gluconeogenesis and PPP activity in preterm infants. In the urinary changes observed during the second week of life, elevated levels of Fructose-1,6-bisphosphate and lactate (Fig. 6B) indicate that metabolic changes in the second week differ from those in the first week. Fructose-1,6-bisphosphate promotes Glycolysis and is involved in the metabolism of Fructose and mannose, representing another overlapping metabolic pathway during the first two weeks of life, in addition to Gluconeogenesis and Glycolysis. Changes in

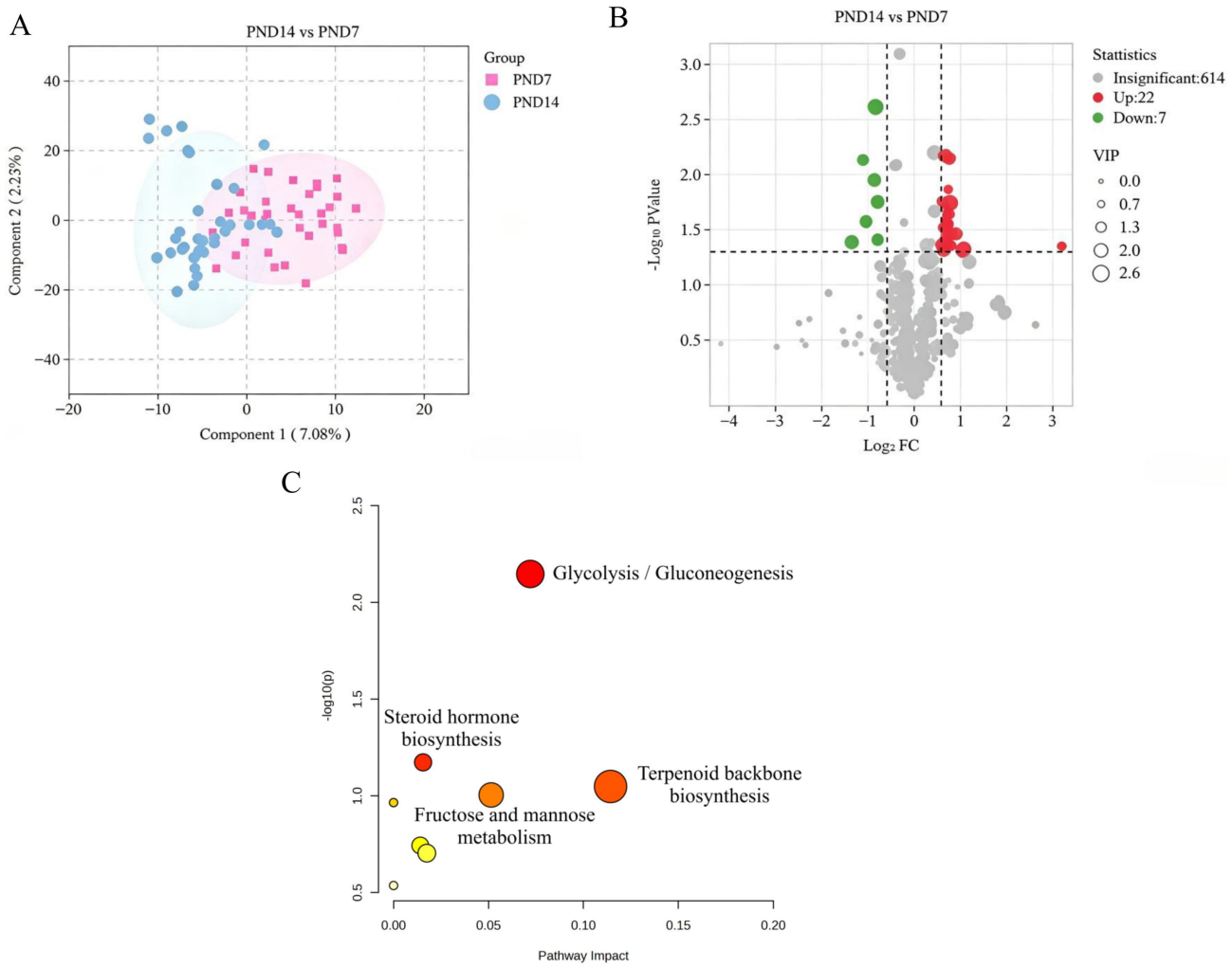

Fig. 4. Analysis of urinary metabolism and enrichment pathways in the second week. Metabolic differences in urine samples from preterm infants between PND14 and PND7: (A) OPLS-DA model for urine samples from PND14 and PND7 ($R^2X = 0.247$, $R^2Y = 0.453$, $Q^2 = 0.418$); (B) Volcano plot of differentially expressed metabolites between PND14 and PND7 urine samples; (C) Enrichment pathway analysis of differentially expressed metabolites in PND14 and PND7 urine samples.

these metabolites and their associated pathways suggest that metabolic adaptation is closely related to the regulation of glycometabolism homeostasis during the early postnatal period in preterm infants.

Discussion

This study aimed to examine the dynamic characteristics of metabolic changes in preterm infants during the early postnatal period. Previous research has already demonstrated that the metabolic profiles of preterm infants differ from those of full-term infants [18]. Recent studies using urinary metabolomics have identified six major biological outcomes affected by preterm birth: nitrogen metabolism, growth, neurochemistry, microbiome metabolism, cellular defense, and metabolic alterations [19]. Most of the observed changes are biologically plausible and consistent with previously reported health complications associated with preterm birth [20]. Therefore, we selected the first two postnatal weeks as the timeframe for longitudinal follow-up in preterm infants to assess whether these metabolic changes are reversible. Through comparative metabolic analysis of urine samples collected on PND1, PND7, and PND14, we obtained several novel and valuable findings. The possible associations between the identified metabolic changes and early adaptation in preterm infants are discussed individually below.

In the urinary metabolism of preterm infants during the first week of life, the levels of most differential metabolites showed an upward trend, particularly among urinary organic acids. Organic acids serve as intermediate products in various metabolic pathways within the body, including glycolysis, the TCA cycle, fatty acid oxidation, ketone metabolism, and coenzyme metabolism [21]. Elevated levels of urinary organic acids in preterm infants indicate metabolic activity in the aforementioned pathways, which

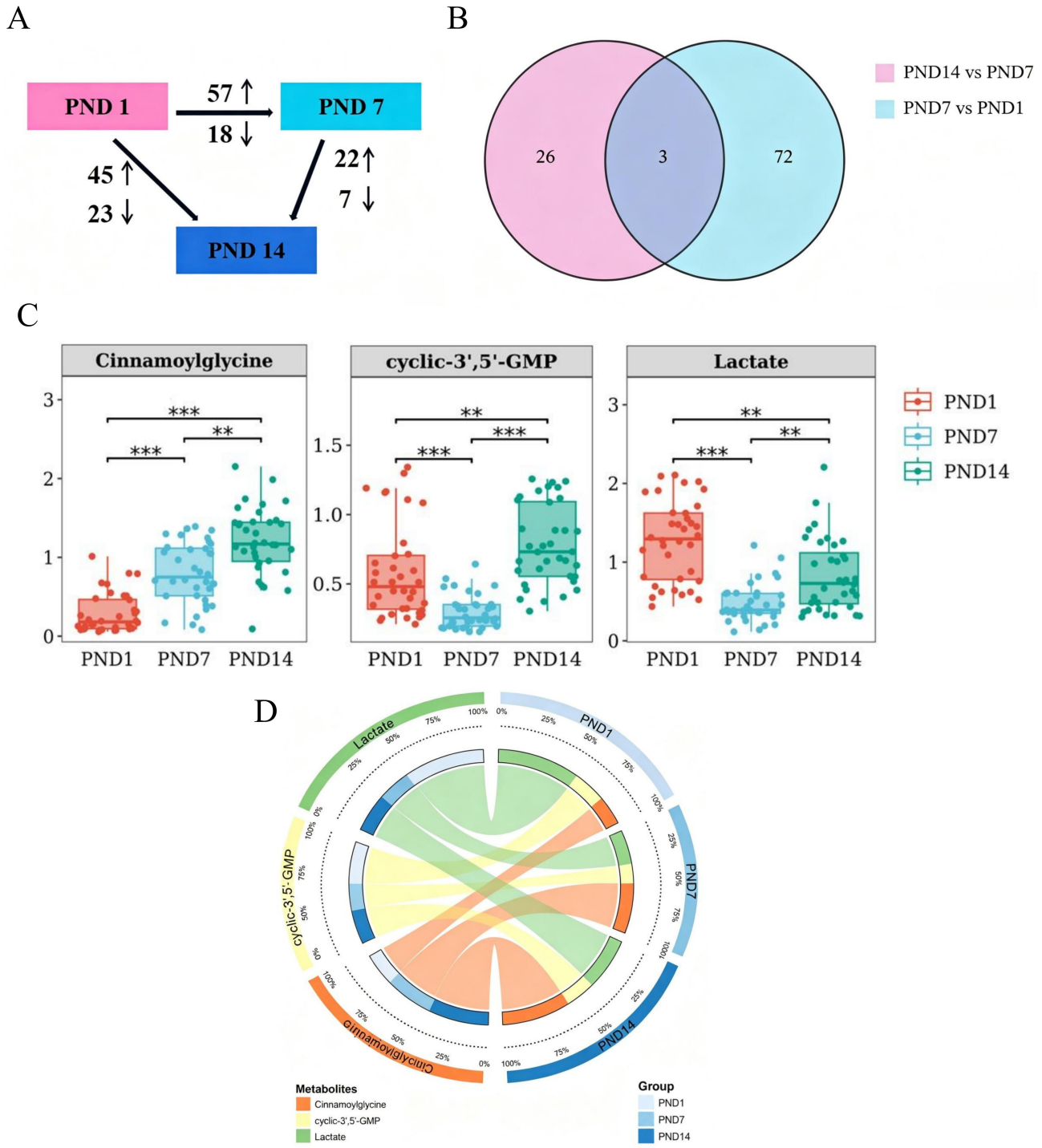

Fig. 5. Dynamic changes in urinary metabolite profiles on PND1, PND7, and PND14. Combined analysis of differential urinary metabolites during the first and second postnatal weeks in preterm infants: (A) Number and changes in differentially expressed urinary metabolites among PND1, PND7, and PND14; (B) Venn diagram showing three common differentially expressed urinary metabolites in preterm infants during the first and second weeks after birth; (C) Box plots showing the difference tests for the three common differential metabolites (cinnamoylglycine, cGMP and lactate) in urine levels at PND1, PND7, and PND14 (**$p < 0.01$, ***$p < 0.001$); (D) Circos diagrams illustrating the distinct metabolic responses of cinnamoylglycine, cGMP and lactate at PND1, PND7, and PND14.

may also be related to the initiation of exogenous nutritional supplementation. Furthermore, genetic single-enzyme defects that block certain metabolic pathways in organic acid metabolism can lead to elevated levels of organic acids in urine, a condition known as organic aciduria. The detection of organic acid concentrations in urine using GC-MS tech-

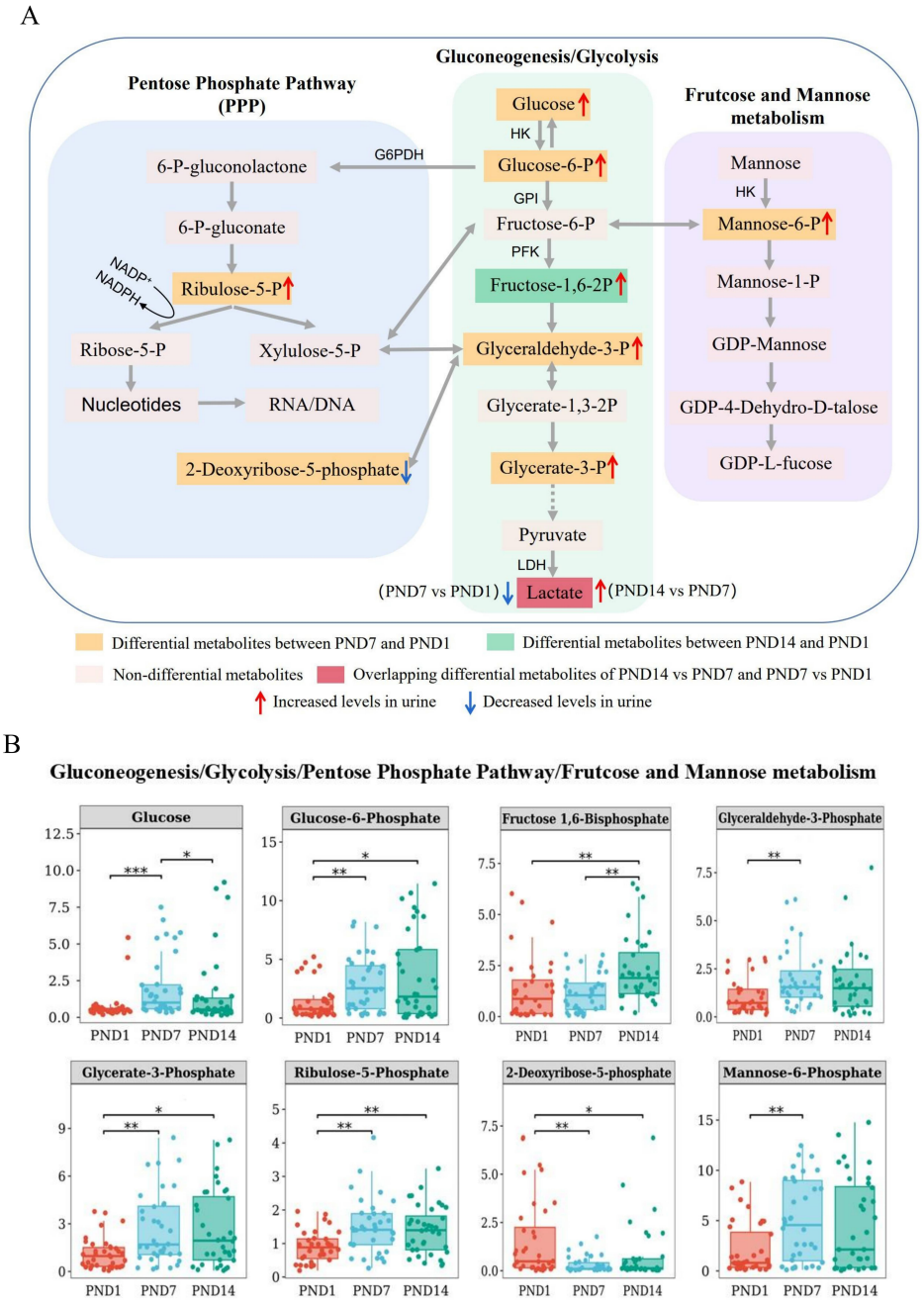

Fig. 6. Comparison of urinary metabolic pathways in preterm infants during the first two postnatal weeks. A comprehensive analysis of significantly enriched urinary metabolic pathways in preterm infants from the first week to the second after birth: (A) Schematic diagram illustrating changes in metabolic pathways associated with differentially expressed metabolites in the urine of preterm infants during the first and second weeks after birth, including Glycolysis/Gluconeogenesis, Pentose phosphate pathway and Fructose and mannose metabolism (yellow indicates differentially expressed metabolites in the urine of preterm infants during the first week after birth. Green indicates differentially expressed metabolites in the urine of preterm infants during the second week after birth, red indicates differentially expressed metabolites common to both weeks; red arrows indicate upregulation in urine levels, and blue arrows indicate downregulation in urine levels); (B) Box plot illustrating the results of a comparison of urinary levels of glucose, Glucose-6-phosphate, Fructose-1,6-bisphosphate, Glyceraldehyde-3-phosphate, Glyceric acid-3-phosphate, Ribulose-5-phosphate, 2-Deoxyribose-5-phosphate, and Mannose-6-phosphate at PND1, PND7, and PND14 (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). HK, hexokinase; GPI, glucose phosphate isomerase; PFK, phosphofructokinase; G6PDH, Glucose-6-phosphate dehydrogenase; LDH, lactate dehydrogenase. (Fig. 6A was created using Microsoft 365 PowerPoint, https://www.microsoft.com/microsoft-365/powerpoint).

nology serves as a key diagnostic tool for many inherited metabolic disorders [22]. In our study, among the urinary organic acids in preterm infants during the first week of life, L-3-HK showed the most pronounced elevation. L-3-HK is a metabolite of the kynurenine pathway, which serves as the primary route for tryptophan degradation in mammals [23]. Existing evidence indicates that L-3-HK generates highly reactive free radicals, participates in oxidative stress reactions, is associated with excitotoxicity, influences cognitive function, and is present at elevated concentrations in the blood of patients with Alzheimer's disease [24,25]. We speculate that the elevated L-3-HK levels in the urine of preterm infants during the first week after birth indicate the possibility of sustained oxidative stress; this also suggests increased metabolism in the canine urea cycle, which may be related to the long-term development of neurocognitive function in preterm infants.

The pentose phosphate pathway (PPP) was one of the most significant metabolic pathways identified in the differential metabolite enrichment analysis of first-week urine samples from preterm infants in this study. PPP provides the pentose (5-phosphoribose) for DNA/RNA synthesis to cells, as well as the necessary NADPH for generating a large amount of energy [26]. NADPH is not only one of the main defenses of cells against oxidative stress through the glutathione system but also provides reducing power for various reactions, particularly lipid synthesis [27,28]. Baquer et al. [29] used ^{14}C-labeled glucose to demonstrate that basal PPP activity is enhanced in the brains of early-stage rat pups and declines with age. The authors also suggested that the developing brain relies on the PPP to support various functions as it progresses through growth and myelination toward full neural functionality. There is also evidence suggesting that glucose-6-phosphate dehydrogenase and the PPP exert neuroprotective effects, primarily by mitigating oxidative stress induced by various factors [30]. In conjunction with our findings, the levels of PPP metabolites in urine were significantly elevated in preterm infants during the first week after birth, indicating heightened PPP activity. We speculate that this is the positive regulatory result of the increased demand for nucleotides and lipids in premature infants in the early stage. This further underscores that the impact of early energy metabolism on brain development in preterm infants cannot be overlooked.

The number and types of differential metabolites in the urine of preterm infants in the second week were significantly fewer than in the first week, indicating that the metabolic profile of preterm infants in the second week after birth had gradually stabilized compared to the first week. Similar to the first week, elevated levels of organic acids remained the primary feature of differential metabolites in the second week, although the specific metabolites involved differed. HPHPA was the metabolite showing the most pronounced increase in the urine of preterm infants during the second week after birth. It has been reported that HPHPA is an abnormal phenylalanine metabolite derived from bacterial metabolism in the gastrointestinal tract, suggesting a potential association between urinary metabolism and the gut microbiota in preterm infants [31]. Elevated levels of HPHPA have been detected in the urine of children with autism spectrum disorder (ASD), although the underlying mechanism remains unclear and may be related to the disruption of catecholamine signaling [32].

Previous studies have demonstrated that preterm infants are prone to hypoglycemia and hyperglycemia in the early postnatal period, and glycometabolismhas consistently been a critical component of metabolic adaptation and homeostasis in preterm infants [33,34]. In this study, Glycolysis/Gluconeogenesis showed significant enrichment of differential urinary metabolites in preterm infants during the second week. Within this pathway, urinary levels of lactate and Fructose-1,6-diphosphate (FDP) were elevated, which contrasts markedly with the PPP observed during the first week.

Although urinary metabolic changes differ between the first and second weeks of life in preterm infants, we have identified some similarities. These shared metabolic characteristics may have unique associations with early postnatal growth and development in preterm infants. Cinnamoylglycine, cGMP and lactate are common differential urinary metabolites within the first 2 weeks of life, while glycolysis and gluconeogenesis are common metabolic pathways. Cinnamoylglycine is a known human urinary metabolite and a secretory solute of the renal proximal tubule [35]. Chen et al. [36] performed a prospective cohort study involving 3416 patients with chronic kidney disease (CKD) and found that reduced renal clearance of solutes such as cinnamoylglycine was significantly associated with an increased risk of CKD progression. Combined with the changes in urinary cinnamoylglycine levels observed in preterm infants within the first 2 weeks of life in our research, it is speculated that cinnamoylglycine may be positively correlated with early renal excretory function in preterm infants. However, further evaluation in conjunction with eGFR and other indicators is required.

There was no clear pattern in the changes in urinary cGMP and lactate levels over these two weeks. cGMP is a unique second messenger molecule produced in various cell types and tissues and targets a wide range of downstream effectors, eliciting a broad spectrum of cellular effects [37]. A previous animal study had shown that plasma cGMP levels rise significantly during the second and third trimesters of pregnancy, accompanied by increased urinary cGMP excretion. This may indicate that cGMP mediates maternal cardiovascular and renal hemodynamic adaptation to pregnancy by dilating blood vessels [38]. In this study, cGMP levels in the urine of preterm infants were also highest on the day of birth and gradually declined, suggesting that cGMP levels in preterm infants during the early postnatal period may be influenced by maternal levels. cGMP

also participates in regulating the cellular phenotype, proliferation, and cytoskeletal dynamics essential for lung development; early postnatal cGMP levels play a facilitative role in establishing a mature pulmonary circulation (adaptation from the intrauterine to the extrauterine environment) [39]. Our findings showed that cGMP levels in the urine of preterm infants at PND7 were lower than those at PND1, which may indicate hypoxic injury caused by immature pulmonary circulation in preterm infants. Although preterm infants typically receive respiratory support and clinical oxygen saturation monitoring, certain metabolic changes within the body may go undetected. As the pulmonary circulation of preterm infants continues to develop and they further adapt to the extrauterine environment, their urinary cGMP levels rebound by PND14. We speculate that urinary cGMP levels may be directly correlated with systemic NO levels and may reflect systemic hypoxic injury in the urine, providing a new approach for the early assessment of pulmonary development and function in preterm infants.

Glycometabolism was the most significant metabolic feature associated with differential urinary metabolites in preterm infants during the first and second weeks after birth. We found that gluconeogenesis was more significantly enriched among urinary differentially expressed metabolites in the first week of life, whereas glycolysis was more prominent in the second week. Lactate, a urinary metabolite common to both time points, exerts a regulatory effect on pathways related to glucose metabolism *in vivo*. Animal studies have demonstrated that lactate strongly inhibits pyruvate dehydrogenase-catalyzed glycolysis and the TCA cycle, but has little effect on glucose utilization in the PPP [40]. Recent evidence suggests that lactate supports long-term synaptic plasticity induced by high-intensity exercise and high-attention-load cognitive tasks, whereas glucose is sufficient to meet the requirements of less demanding neural processing and learning activities [41]. In this study, the relationship between urinary lactate levels in preterm infants and changes in the activity of glycometabolism-related pathways provides partial evidence for the aforementioned regulatory role of lactate. Thus, lactate serves as a pivotal regulator of glucose metabolism-related pathways in preterm infants during the early postnatal period and plays a crucial role in maintaining glucose homeostasis and energy metabolism.

The primary limitation of this study is that the effects of exogenous factors on urinary metabolism were not considered. Gestational age, birth weight, gender, and mode of delivery may all influence early urinary metabolism; the lack of analysis of these factors may have introduced bias into our results. In particular, differences in urinary metabolism potentially associated with postnatal feeding with breast milk or formula should be a focus of our future research. Second, this study was conducted at a single center, involved a small sample size, and lacked a control group of full-term newborns. Consequently, our interpretation of biochemical maturity is limited to the context of preterm infants and describes only the general patterns of urinary changes in preterm infants during the early postnatal period. These limitations may also explain why the predictive models used in this study did not perform as well as expected, and indicate directions for improving future research. Further investigation is needed to identify key molecules involved in early metabolic regulation in preterm infants and to validate these findings in larger-scale studies, thereby laying the foundation for the clinical application of specific metabolic signals as diagnostic and prognostic markers.

Conclusion

This study describes the general characteristics of urinary metabolomic changes in preterm infants during the first two weeks after birth. We found that these changes were more pronounced during the first week than the second week, suggesting that metabolic adaptation in preterm infants may primarily occur during the first week after birth. Glycometabolism is a key factor in the urinary metabolic changes observed in preterm infants during the first two weeks after birth, providing a reference for metabolic adaptation and clinical management strategies in the early postnatal period.

Availability of Data and Materials

The data used to support the findings of this study are available from the corresponding author upon request.

Author Contributions

HX and HRT designed the research study; SH, QS and YHZ recruited subjects; SH and FJW collected urine samples; SH and XYM performed the research. SH and TYL analyzed the data and draft the manuscript. QS and YHZ contributed to data analysis and manuscript preparation. FJW contributed to manuscript revisions and data analysis. All authors contributed to important editorial changes in the manuscript. All authors gave final approval of the version to be published. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study is in accordance with the Declaration of Helsinki. The study was approved by the Ethics Committee of the Children's Hospital Affiliated of Fudan University (No. 2020-524). The principle of informed consent was followed throughout the experiment, and information about the study was provided to patients or their families, and consent was obtained.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.24976/Discov.Med.202638211.189.

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