Original Research

Metabolic analysis of infants with bronchopulmonary dysplasia under early nutrition therapy: An observational cohort study

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Impact statement

Bronchopulmonary dysplasia (BPD) is a multifactorial and complex disorder. Nutrition and metabolic changes have been proposed to participate in the occurrence of BPD. However, metabolic patterns of major nutrients in different nutritional stages and their role in the pathophysiology of BPD are blurred. Our metabolic study showed seven significant amino acids (AAs) and carnitines capable of distinguishing BPD infants, simultaneously identified the Gln/C6:1 ratio as a potential indicator of BPD. Our work demonstrates that the metabolic dysregulation of AA and carnitine profiles are responsible for the development of BPD. These results may enhance the cognition about the pathogenesis of BPD and provide a prospective therapy targeting for BPD.

Abstract

To assess the amino acid and fatty acid metabolite patterns between infants with and without bronchopulmonary dysplasia in different nutritional stages after birth and identify metabolic indicators of bronchopulmonary dysplasia. This was an observational cohort of preterm infants born at a gestational age $\leq 32+6$ weeks and with a body weight ≤ 2000 g. Amino acid and carnitine profiles were measured in dried blood spots (DBSs) during the early nutrition transitional phase using tandem mass spectrometry. Bronchopulmonary dysplasia was defined as oxygen dependence at 36 weeks of postmenstrual age or 28 days after birth. Metabolomic analysis was employed to define metabolites with significant differences, map significant metabolites into pathways, and identify metabolic indicators of bronchopulmonary dysplasia. We evaluated 45 neonates with and 40 without bronchopulmonary dysplasia. Four amino acids and three carnitines showed differences between the groups. Three carnitines (C0, C2, and C6:1) were high in the bronchopulmonary dysplasia group mostly; conversely, all four amino acids (threonine, arginine, methionine, and glutamine (Gln)) were low in the bronchopulmonary dysplasia group. Pathway analysis of these metabolites revealed two pathways with significant changes ($p < 0.05$). ROC analysis showed Gln/C6:1 at total parenteral nutrition phase had both 80% sensitivity and specificity for predicting the development of bronchopulmonary dysplasia, with an area under the curve of 0.81 (95% confidence interval 0.71–0.89). Amino acid and fatty acid metabolite profiles changed in infants with bronchopulmonary dysplasia after birth during the nutrition transitional period, suggesting that metabolic dysregulation may participate in the development of bronchopulmonary dysplasia. Our findings demonstrate that metabolic indicators are promising for forecasting the occurrence of bronchopulmonary dysplasia among preterm neonates.

Keywords: Bronchopulmonary dysplasia, amino acid, carnitine, preterm, metabolomics, nutrition

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Introduction

Bronchopulmonary dysplasia (BPD) is associated with severe neurological impairment and remains a common morbidity of prematurity.[1,2] The incidence of BPD in 2010 was reported to be 42% in premature infants from 22 to 28 gestational weeks (GW) based on the traditional definition of supplemental oxygen use at 36 weeks.[3]

BPD is a multifactorial and complex disorder, and multiple etiological studies have focused on the predisposing factors. Recent research on the role of nutrition and metabolic changes has been carried out. A study of 296 infants

born before 28 GW demonstrated that early acquisition of energy and protein was correlated to less risk for BPD.[4] Nutrition is vital to promote organ development and avoid maldevelopment by providing major nutritional substrates, including amino acids (AAs) and fatty acids (FAs). The catabolism of crucial AAs and FAs provides substrates for oxidative phosphorylation to generate energy in mitochondria, as well as yield abundant functional metabolites for cellular structure and biosynthesis. Metabolic homeostasis is critical for maintaining cellular activities under physiological and pathological conditions[5]; however, metabolic dysregulation may disturb regular cellular bioenergetics such as proliferation, differentiation, and apoptosis, which were deemed to participating in chronic lung diseases.[5–7] Most preterm infants, especially those born < 32 GW, experience nutritional therapy involving initial parenteral nutrition (PN) and subsequent transition to full enteral nutrition (EN). Due to various intake methods and metabolic efficiency, there are changes in the metabolic AA patterns across the different nutrition transitional phases in our subject.[8] Recent studies have suggested metabolic dysregulation may be involved in the etiology of chronic lung disease, including BPD.[9–12] However, the metabolic patterns of major nutrients in different nutritional stages and their role in the pathophysiology of BPD are not fully understood.

Metabolomics is a promising approach for the quantitative analysis of low-molecular-weight metabolites such as AAs and carnitines, which are catabolized from major nutrients. It can be used to distinguish metabolite profiles between infants with and without BPD and allows for the identification of metabolic indicators specific to BPD. Therefore, we performed a metabolomics study of AAs and carnitines on dried blood spots (DBSs) quantified by tandem mass spectrometry (MS/MS) in prematurity born before $32 + 6$ GW and weighing $\leq$2000 g at four different nutritional stages, aiming to identify significantly different metabolites and explore specific metabolic predictors of BPD during the nutrition transition phase.

Materials and methods

Data collection

The data were collected from the observational cohort "a prospective study of amino acids profiles in premature infants receiving nutritional support," which was registered on https://www.clinicaltrials.gov/ (NCT03100305) and approved by the medical ethics committee of Xinhua Hospital (XHEC-2016–139). The registry was conducted in preterm infants hospitalized at the neonatal intensive care unit (NICU) of Xinhua Hospital affiliated to Shanghai Jiao Tong University School of Medicine from December 2016 to December 2017. In the current study, preterm infants born at $\leq 32 + 6$ GW and with a BW $\leq$2000 g were recruited for metabolomic analysis of AAs and carnitine profiles, and those with genetic disorders, digestive congenital anomalies, discharge, or died before achieving target enteral feeding were excluded. The parents of all enrolled infants were informed about the study and signed the written consent in advance.

The following clinical data were collected: maternal history, including gestational hypertension (GH) or eclampsia, gestational diabetes mellitus (GDM), intrauterine distress, infant fetal growth retardation, antenatal infection, and corticosteroid administration; delivery history including GA, BW, GW Z-score, and Apgar scores, and so on; complications including intraventricular hemorrhage grades 3–4, extrauterine growth restriction (EUGR) at discharge, neonatal respiratory distress syndrome (NRDS), pulmonary hypertension (PH), intraventricular hemorrhage (IVH), patent ductus arteriosus (PDA); and treatment and nutritional support, including oxygen use and mechanical ventilation (MV), initial age of minimal enteral nutrition (MEN), and EN. The final outcome was the presence of BPD; infants were assigned into BPD and no BPD groups accordingly. Birth weight Z-scores were calculated using the Fenton method.[13] BPD was defined as supplemental oxygen or respiratory support at 36 weeks of postmenstrual age or 28 days after birth, as proposed by the National Institute of Child Health and Human Development (NICHD).[14]

The nutrition regimens were formulated according to the 2013 Chinese guidelines for neonatal nutrition therapy. PN was introduced with an initial rate of 80 mL/kg/day, increasing at 20 mL/kg/day, and up to the goal of 150–160 mL/kg/day. AAs (Pediatric Compound Amino Acid injection 18AA-II, PAA 6%; Treeful, Shanghai, China) were initiated at 1.5–2 g/kg/day, increasing by 0.5–1 g/kg/day to the target of 3.5–4.0 g/kg/day, while lipid infusion (Lipofundin MCT/LCT, 20%; Braun Medical, Melsungen, Germany) were initiated at 1 g/kg/day, increasing by 0.5–1 g/kg/day until the goal of 3 g/kg/day. Breast milk (if available) or, alternatively, formula milk were supplied after birth as tolerated; EN was initiated at 20 mL/kg/day and increased to 60–180 mL/kg/d gradually. MEN was defined as a minimal volume $<$20 mL/kg/day. Energy intakes in EN were calculated at 85% discount.

During the transition from PN to EN, DBSs were collected dynamically from all enrolled preterm neonates at four time points. The regimen of specimen collection is described in detail in previously published manuscript.[8] In brief, sampling points were as follows: point 1, $<$24 h after birth; point 2, as AA intake reaching the goal or maximum before EN started; point 3, when the EN/(EN + PN) energy intake ratio reached about 50%; and point 4, when full EN was achieved. A 20% missing data rate was allowed at each point; however, we excluded cases with less than three DBS samples. The DBS samples were sealed in zip plastic bags and stored at –20°C. Subsequently, they were extracted, eluted with a linear gradient, and then analyzed using MS/MS (Xevo-TQ, Waters, Milford, MA). Eighteen AAs and 40 carnitines were tested using the multiple reaction monitoring mode. The standard curve method was used for further quantitative analysis. The 18 detected AAs were the following: threonine (Thr), phenylalanine (Phe), arginine (Arg), leucine (Leu), methionine (Met), valine (Val), citrulline (Cit), tryptophan (Trp), tyrosine

(Tyr), aspartic acid (Asp), proline (Pro), glutamine (Gln), alanine (Ala), glutamate (Glu), ornithine (Orn), glycine (Gly), serine (Ser), and histidine (His). The tested carnitines included two free carnitines (C0 and C2) and the remaining 38 acyl-carnitines (ACs).

Statistical analysis

All analyses were performed using SPSS 22.0 (IBM Corp., Armonk, NY) primarily. The normality analysis was used Shapiro-Wilk test. Differences between infants with and without BPD were evaluated by univariate analysis such as t-test, U-test, or the Chi-square test, as appropriate. Simultaneously binary logistic regression analysis was employed to verify the independent correlation of metabolites and significant variables on univariate analysis with the risk of BPD. Significance was stated as $p < 0.05$. The concentration of AAs and carnitines (unit: μM) obtained by MS/MS analysis was preprocessed with normalization, essential transformation, and scaling, then imported into MetaboAnalyst 4.0 (Wishart Re-search Group, University of Alberta, Edmonton, Canada) for further analysis. Then we established principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) between the BPD and no BPD groups. Significantly different metabolites were identified as variable importance in the projection (VIP) >1 in PLS-DA model and $p < 0.05$ in univariate analysis. They were then imported into the Kyoto Encyclopedia of Genes and Genomes for pathway enrichment analysis, and the significant pathways were selected if p was <0.05 and pathway impact (PI) >0.1. Receiver operating characteristic (ROC) analysis was utilized to identify significant metabolic indicators that provided optimal sensitivity and specificity for the risk of BPD.

Results

Characteristics of preterm infants

A total of 85 preterm infants were included, of which 45 developed BPD, and 304 DBS samples were collected as follows: 64 were collected at point 1, 82 at point 2, 84 at point 3, and 74 at point 4. Worsened clinical status and unexpected discharge prior to the target volumes of EN caused a smaller sample size at points 1 and 4, respectively. BPD infants were more immature, with significantly low GA and BW. Infants who developed BPD had a longer oxygen support and MV duration, as well as a higher incidence of EUGR at discharge. It was comparable between the two groups in sex, mode of delivery, BW Z-score, Apgar

Table 1. Clinical characteristics of enrolled infants.

	BPD group (n = 45)	No BPD group (n = 40)	χ^2/U/t	p
GA (w)	29.11 (25.80, 32.24)	32.00 (30.44, 32.71)	–6.47	<0.01**
BW (g)	1275 (833, 1732)	1680 (1257, 1909)	–6.40	<0.01**
BW z-score	0.09 (–1.56, 0.98)	–0.23 (–1.09, 0.65)	–1.55	0.12
SGA	2	0	2.58	0.11
Sex (n)			0.15	0.70
Male	31	26		
Female	14	14		
Cesarean delivery (n)	23	27	2.35	0.13
Apgar at 1 min	9 (4, 10)	9 (4, 10)	–1.59	0.11
Apgar at 5 min	10 (6, 10)	10 (7, 10)	–0.25	0.80
GH or eclampsia (n)	5	7	0.71	0.39
GDM (n)	8	3	1.18	0.28
Fetal distress (n)	6	7	0.28	0.59
FGR (n)	2	1	0.24	0.62
Antenatal infection (n)	2	4	1.01	0.31
Postnatal corticosteroids (n)	2	4	1.01	0.31
Severe IVH (III–IV, n)	2	0	2.58	0.91
EUGR at discharge (n)	30	15	7.23	<0.01**
NRDS (n)	10	9	0.00	0.97
PH (n)	11	10	0.71	0.39
PDA (n)	2	3	0.35	0.55
Sepsis (n)	8	2	2.21	0.14
Mild BPD (n)	/	21		
Moderate/severe BPD (n)	/	24		
MV (n)				
Duration of oxygen (d)	46 (28, 90)	10 (0, 26)	–7.92	<0.01**
Duration of MV (d)	3 (10, 45)	0 (0, 9)	–3.83	<0.01**
Start age of PN (h)	26 (7, 48)	24 (6, 31)	–0.75	0.44
Start age of MEN (d)	4 (1, 12)	2 (1, 9)	–5.44	<0.01**
Start age of EN (d)	7 (2, 19)	4 (2, 11)	3.22	0.01*

Note: Clinical characteristics data were collected from infants with BPD (n = 45) and those without BPD (n = 40). Data are represented as numbers, mean ± standard deviation, or median/interquartile range (IQR), respectively.
BPD: bronchopulmonary dysplasia; GA: gestational age; BW: birth weight; SGA: small for gestational age; GH: gestational hypertension; GDM: gestational diabetes mellitus; FGR: fetal growth retardation; IVH: intraventricular hemorrhage; EUGR: extrauterine growth restriction; NRDS: neonatal respiratory distress syndrome; PH: pulmonary hypertension; PDA: patent ductus arteriosus; MV: mechanical ventilation; PN: parenteral nutrition; MEN: minimal enteral nutrition; EN: enteral nutrition. *$p < 0.05$, **$p < 0.01$.

scores at 1 and 5 min, maternal GH and GDM, antenatal steroid use and infection, severe IVH, PH, PDA, NRDS, and sepsis (Table 1). Furthermore, infants with BPD showed delayed MEN and EN (Table 1); however, both groups had comparable average intakes of AAs, FAs, glucose, energy, and fluid volume at different nutritional stages (Table 2).

Metabolites with differences between infants with BPD and without BPD

To identify metabolites with differences between the BPD and no BPD groups, PCA and PLS-DA models were successively performed on all metabolites using MetaboAnalyst 4.0. The PLS-DA showed various metabolic profiles and a tendency for separation between the samples from the BPD and the no-BPD groups at the four different nutritional phases: infants in BPD group diffusely distributed on the left square (red triangle), while infants in no-BPD group were located on the right side (green cross) with small overlap with BPD group (see Figure 1).

To define the metabolic predictors contributing to distinguishing infants with and without BPD, the VIP scores were counted for each metabolite at four different nutritional phases. Seventeen AAs and three carnitines with VIP score >1 were identified as candidates: C0, Thr, His, Ser, Met, Phe, C2, and Val at Point 1; Gln, C0, Met, Arg, Glu, Phe, Trp, Gly, C2, and His at Point 2; Gly, Met, Val, Thr, Pro, Ala, Trp, Leu, C2, Tyr, and C0 at Point 3; and Thr, Arg, Asp, Leu, Glu, Tyr, Pro, Val, Orn, and C6:1 at Point 4. To further narrow the scope of candidate metabolites to differentiate the BPD and no-BPD groups, we employed univariate analysis on these 22 candidates, and obtained seven metabolite predictors including four amino acids and three carnitines: Arg, Gln, Met, Thr, C0, C2, and C6:1. Compared to the no BPD group, infants in the BPD group had lower levels of Thr, Met, Gln, and Arg at various nutritional phases, as well as higher levels of most carnitines: Thr (at Point 1,3 and 4), Gln and Arg (at Point 2), as well as Met (at Point 3) were decreased in BPD group. C0 (at Point 1and 2), C2 (at Point 2) and C6:1 (at Point 3) were increased, while C0 at Point 3 were decreased in no-BPD group. All identified biomarkers are summarized in Table 3.

Pathway analysis of metabolites with differences

To gain a better understanding of the interaction between various metabolites involved in the pathology of BPD, pathway analysis of seven selected significant metabolites was performed. The results mapped a cluster of metabolites in two significant pathways ($p < 0.05$ and PI >0.1): Ala, aspartate, and Glu metabolism, as well as cysteine and Met metabolism (Table 4 and Figure 2(a)). The detailed diagram of each pathway is shown, and Gln and Met are the most notable metabolites (Figure 2(b) and (c)).

Metabolic indicator of BPD

Binary regression analysis showed that only the Gln level at Point 2 was associated with BPD after adjusting for GA and BW (odds ratio (OR) = 0.69, $p < 0.05$; Table 5). To test the metabolic predictors of the development of BPD, we applied the ROC curves for all identified metabolites in MetaboAnalyst 4.0; the results are shown in Table 6. Gln/C6:1 at Point 2 had optimal sensitivity (80%) and specificity (80%) with a cut-off value of 0.27 for predicting the development of BPD, with an area under the curve of 0.81 (95% confidence interval 0.71–0.89) (Figure 3).

Discussion

In this study, preterm neonates born at $\leq 32+6$ GW and with a BW ≤ 2000 g were enrolled from December 2016 to

Table 2. Nutritional intake at each sampling point in the BPD and no BPD groups.

	BPD	No BPD	Z	p
Point 2				
Average FAs in PN (g/kg/d)	1.87 (1.45, 2.31)	1.79 (1.20, 2.16)	−1.64	0.09
Average AAs in PN (g/kg/d)	2.50 (1.88, 2.81)	2.42 (1.62, 2.78)	−1.07	0.28
Average energy in PN (kcal/kg/d)	62.29 (47.96, 68.91)	53.82 (40.53, 67.79)	−2.01	0.05
Average fluid in PN (mL/kg/d)	100.67 (75.00, 113.99)	96.57 (66.04, 110.30)	−1.50	0.13
Point 3				
Average FAs in PN (g/kg/d)	1.02 (0.76, 1.38)	0.97 (0.67, 1.32)	−1.08	0.27
Average AAs in PN (g/kg/d)	1.31 (1.06, 1.73)	1.25 (1.00, 1.70)	−0.63	0.52
Average energy in PN (kcal/kg/d)	31.51 (25.69, 43.97)	29.69 (24.42, 41.01)	−0.77	0.44
Average fluid in PN (mL/kg/d)	51.57 (44.15, 70.87)	52.72 (40.72, 70.50)	0.15	0.87
Average FAs in EN (g/kg/d)	3.36 (2.17, 4.22)	3.46 (2.85, 3.92)	−0.52	0.60
Average AAs in EN (g/kg/d)	1.77 (1.00, 2.12)	1.82 (1.50, 2.04)	−1.48	0.13
Average energy in EN (kcal/kg/d)	64.58 (39.99, 79.05)	66.70 (54.81, 75.09)	−0.80	0.41
Average fluid in EN (mL/kg/d)	90.25 (56.66, 109.41)	91.70 (75.18, 105.30)	−0.41	0.67
Point 4				
Average FAs in EN (g/kg/d)	4.82 (3.94, 5.42)	4.84 (4.12, 5.25)	−0.17	0.86
Average AAs in EN (g/kg/d)	2.50 (1.79, 2.87)	2.55 (2.17, 2.76)	−0.22	0.82
average energy in EN (kcal/kg/d)	91.85 (72.66, 101.15)	92.84 (79.48, 100.94)	−0.42	0.67
Average fluid in EN (mL/kg/d)	125.10 (104.18, 137.10)	127.63 (112.21, 138.28)	−0.78	0.43

Note: Nutritional intakes were collected at each sampling point from parental to enteral nutrition: Point 2, indicating total PN; point 3, indicating partial EN +PN; and point 4, indicating full EN. Data are represented as median/interquartile range (IQR).
BPD: bronchopulmonary dysplasia; FA: fatty acid; AA: amino acid; PN: parenteral nutrition; EN: enteral nutrition. *$p < 0.05$.

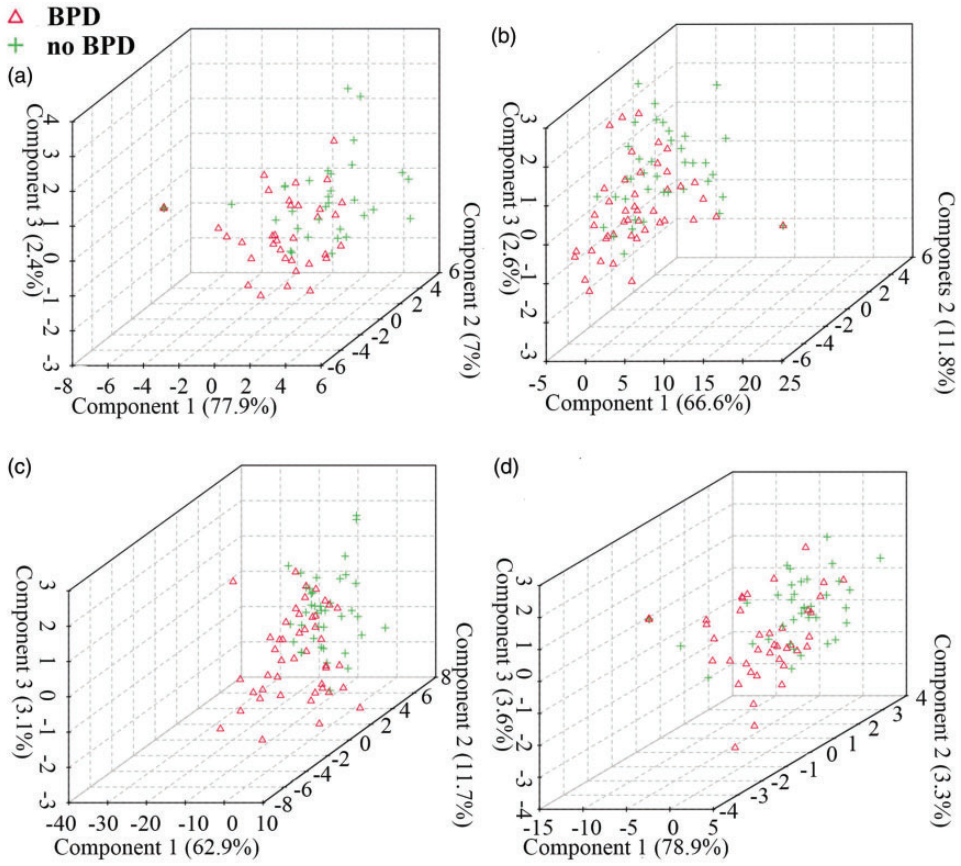

Figure 1. 3D scores with PLS-DA visualization of metabolism profiles between BPD and no BPD group. (a) to (d), representing PLS-DA visualization between the two groups at different nutrition phases from points 1 to 4. The red triangle and green cross represent individual samples, and the distinct clustering of BPD samples separated from the no BPD group to various degrees.
PLS-DA: partial least squares-discriminant analysis; BPD: bronchopulmonary dysplasia.

Table 3. Significant metabolites identified by the PLS-DA model.

	VIP	t-test		p	Trend
		BPD	no BPD		BPD vs. no BPD
Point 1					
C0	3.05	43.57 ± 10.30	35.14 ± 10.47	<0.01**	↑
Thr	2.76	58.38 ± 18.00	71.23 ± 26.95	0.04*	↓
Point 2					
Gln	2.66	4.15 ± 4.39	7.49 ± 4.96	<0.01**	↓
C0	2.58	32.63 ± 11.42	27.44 ± 15.58	0.02*	↑
Arg	2.29	9.84 ± 1.01	18.07 ± 1.92	0.04*	↓
Point 3					
Met	2.09	23.95 ± 6.65	27.08 ± 6.11	0.02*	↓
Thr	1.83	62.17 ± 19.03	73.75 ± 12.24	0.02*	↓
C2	1.24	10.63 ± 3.78	8.41 ± 3.28	<0.01**	↑
C0	1.01	17.62 ± 8.01	20.66 ± 7.69	0.04*	↓
Point 4					
Thr	2.76	62.70 ± 20.18	75.65 ± 26.29	0.03*	↓
C6:1	1.03	0.36 ± 0.042	0.03 ± 0.01	<0.01**	↑

Note: Significant metabolites were identified by the PLS-DA model between infants with BPD and those without BPD at four sampling points from parental to enteral nutrition: Point 1, indicating pre-PN; Point 2, indicating total PN; point 3, indicating partial EN +PN; and point 4, indicating full EN.
PLS-DA: partial least squares discriminant analysis; VIP: variable importance in the projection; BPD: bronchopulmonary dysplasia. VIP>1.0 and *p <0.05 or **p <0.01, significant difference between groups; ↑ and ↓: higher or lower levels of metabolites in infants with BPD than those without BPD.

December 2017. Therefore, we adopted the definition of BPD proposed by NICHD in 2001, rather than the revised definition of BPD in 2018.[15] The incidence of BPD in this study was 53%, which is in agreement with previous studies.[3] Preterm infants with BPD had lower average GA and BW; they also tended to need longer courses of MV therapy and to have delayed intestinal feeding than those without BPD. The results were consistent with those of

Table 4. Results of pathway analysis using MetaboAnalyst 4.0.

Metabolic pathway	Metabolites involved	PI	p
Alanine, aspartate, and glutamate metabolism	28	0.11	0.02*
Cysteine and methionine metabolism	33	0.11	0.04*

Note: Pathway analysis of metabolites with differences was performed and mapped 2 significant pathways.
PI >0.1: a significant pathway; PI: pathway impact. *p < 0.05 and PI >0.1, significant difference between groups.

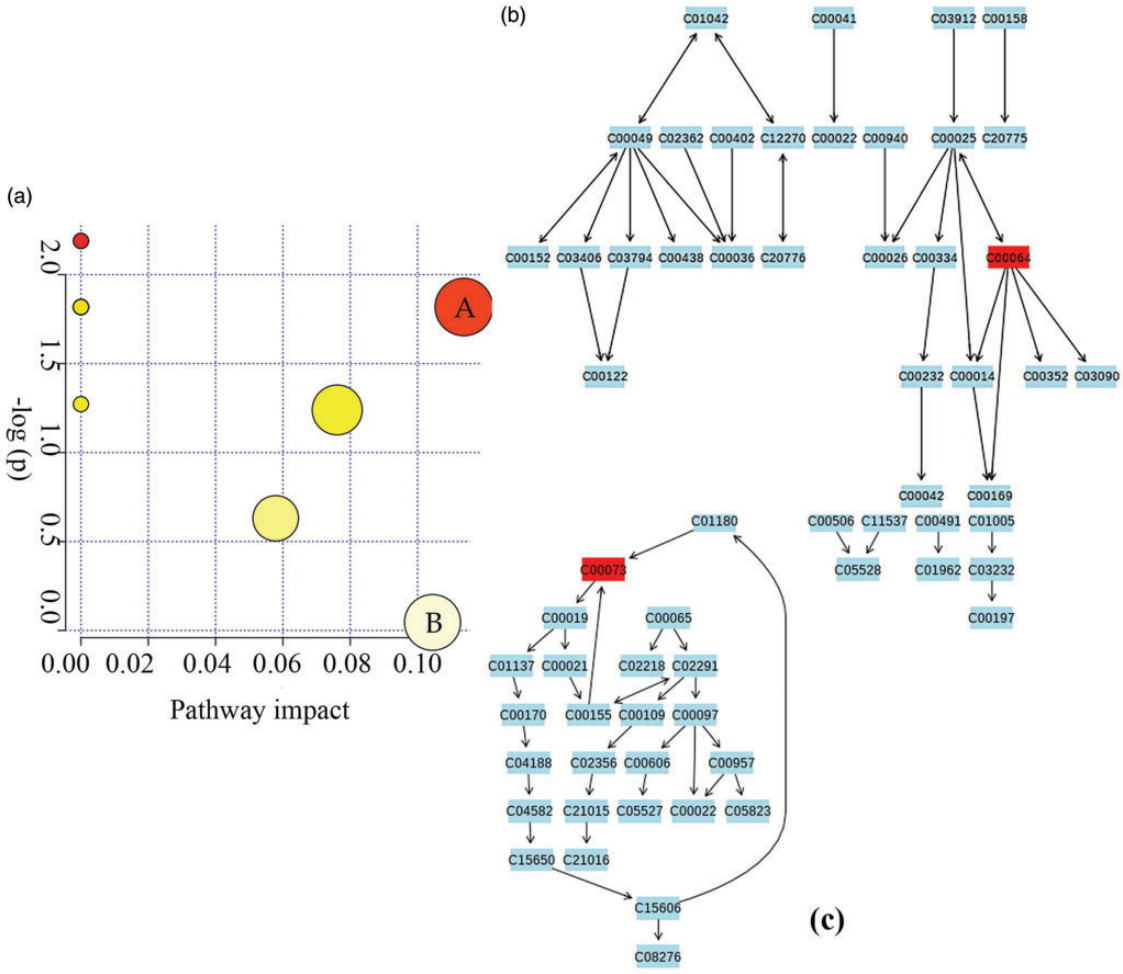

Figure 2. The overview of significant metabolites using pathway analysis. (a) The bubble chart was constructed with PI and p value of all matched pathways, which are expressed as circles with different colors and sizes. It mapped all significant metabolites to seven pathways, two of which were significant (PI >1): A, alanine, aspartate, and glutamate metabolism; and B, cysteine and methionine metabolism. (b) and (c): The representation of the two significant pathways. The involved metabolites were named according to the Kyoto Encyclopedia of Genes and Genomes ID, and the strength was labeled with different colors: red for higher and blue for lower strength. Gln (c00064) and Met (c00073) were the most notable metabolites.
PI: pathway impact.

Table 5. Results of binary logistic regression for BPD.

	p	OR	95% CI
GA	0.04	0.27	0.08–0.95
BW	0.01	0.99	0.98–0.99
Gln at Point 2	0.03	0.69	0.50–0.96

Note: Binary regression analysis was employed to examine the association between BPD and GA, BW, and Gln level.
BPD: bronchopulmonary dysplasia; GA: gestational age; BW: birth weight; OR: odds ratio; 95% CI: 95% confidence interval.

previous studies.[4,16] The delayed introduction of MEN and EN in infants with BPD may have been caused by an unstable clinical status, low GA, and the clinician's decision.

To avoid confounding, we compared the metabolic profiles of neonates with a similar nutritional status. Moreover, previous reports have shown that metabolic abnormalities may persist in the lungs of infants with BPD, suggesting the importance of conducting longitudinal studies to monitor metabolic changes among premature infants.[17,18] Thus, we

Table 6. ROC analysis of significant metabolites for predicting the development of BPD.

Time points	Metabolites	AUC
Point 2	Glutamine/C6:1	0.81
Point 4	C6:1	0.73
Point 3	Met/C6:1	0.69
Point 2	Glutamine	0.69
Point 2	Glutamine/Gly	0.68
Point 3	Gly	0.58
Point 3	Met/Gly	0.57
Point 2	Met/Glutamine	0.56
Point 3	Met	0.54

Note: ROC analysis was used to identify significant metabolic indicators for BPD.
ROC: receiver operating characteristic; BPD: bronchopulmonary dysplasia; AUC: area under the curve.

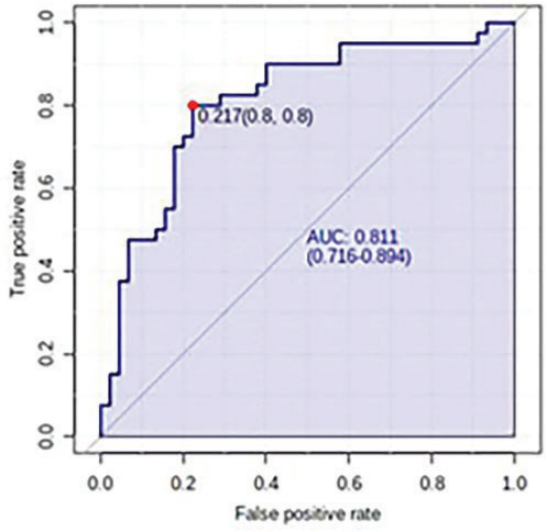

Figure 3. The ROC curve of Gln/C6:1 at point 2 in MetaboAnalyst 4.0. AUC = 0.81; the shaded areas represent a 95% confidence interval of 0.72–0.89 and the solid red dot is the cut-off value of 0.22, with 80% sensitivity and specificity.
ROC: receiver operator characteristics; AUC: area under the curve.

collected samples at four different nutritional phases during the transition from PN to EN as described by Miller *et al.*[19] DBSs were selected because of their low invasiveness and easy access. This study showed that there were significant metabolic differences in AAs and carnitine profiles between infants with and without BPD during the transition between nutritional methods. Four AAs and three carnitines were identified; two major metabolic pathways were also screened.

Metabolic homeostasis affects multiple cellular bioenergetic processes such as cell proliferation, differentiation, autophagy, and apoptosis, which were involved in the development of chronic lung diseases, including BPD.[7,20,21] It has been shown that infants with BPD have elevated metabolite substrates and reduced L-type amino acid transporter-1 levels,[21,22] suggesting the presence of abnormal metabolism, which in turn has been associated with a dysfunction of mitochondrial respiration in hyperoxia induced lung injury *in vitro* or in BPD animal models.[23,24] This study identified the levels of four AAs, Arg, Gln, Met, and Thr, which were significantly lower in infants with BPD. Current studies have exhibited that plasma Arg levels were low in an animal model of BPD,[24–27] but not in BPD infants[28]; however, indirect supplementation of L-Arg in plasma would attenuate arrested alveolar growth through involvement in the release of nitric oxide.[29] Gln is oxidative fuel source for rapidly dividing cells, and extensively involved in bioenergetic processes, such as mitochondrial respiration through the tricarboxylic acid cycle.[30] In addition, it contributes to the biosynthesis of several non-essential AAs and bioactive substances such as glutathione, which is fatal in regulating lung development and hyperoxia responses in the development of BPD.[31] The meta-analysis, which enrolled 12 randomized controlled trials, did not find any effect of Gln supplementation on mortality or major neonatal morbidities including invasive infection and necrotizing enterocolitis in 2877 preterm infants.[32] Besides, Brown *et al.* raised that Gln supplementation may be beneficial for young infants with severely compromised metabolism and Gln availability is rate limiting for tissue repair.[33] *In vitro*, Gln deprivation may worsen cell death caused by hyperoxia[34]; moreover, Gln supplementation could promote mitochondrial function and attenuate hyperoxia-induced acute pulmonary injury in mice.[34,35] Yet, no study was conducted about the effects of Gln supplementation on BPD in preterm infants. In our study, lower plasma Gln was observed in infants with BPD at total PN phase during first days after birth, which may be caused by the following reasons: first, no routine parenteral Gln was supplemented due to its instability in aqueous solution, then provision of EN was generally delayed which may disturbed acquisition of enteral Gln. In the end, Gln consumption rate exceeded supply during catabolic stress in severely ill preterm infants. Considering the relative Gln deficiency in preterm infants with BPD and its protective effect in lung injury model, further evaluation of Gln supplementation in preterm infants with BPD may be promising. Besides, Met is a critical cellular antioxidant, and it can be converted into cysteine, which may replenish intracellular Gln stores. However, there are only a few studies about the role of Met and Thr in the pathogenesis of BPD.

FAs, another major nutrient, were observed to be dysregulated by hyperoxia exposure in neonatal rat models and epithelial cells of rat lungs.[11] Carnitines, including free (C0 and C2) and ACs, are biomarkers of FA metabolism, with free carnitines assisting FAs in generating ACs and transporting them into mitochondria for further bioenergetics through β-oxidation. Assessment of their patterns in infants with BPD may reveal changes in FA metabolism associated with the pathogenesis of BPD. It has been noted that supplementation with L-carnitine could attenuate hyperoxia-induced apoptosis and lung injury in the animal model.[11] Peterson *et al.* noted that neo-natal hyperoxia reduced levels of carnitines and ACs, especially those with long chains at seven days after birth in mice lungs.[12] In this study, no decrease in carnitines was observed in infants with BPD except for C0 at point 3. Despite these similar results, they still demonstrated that there was

dysregulation of FA metabolism during abnormal lung development in infants with BPD and animal models. The fluidity of the metabolome in response to different clinical statuses allows us to establish a metabolism analysis according to the changes in AA and carnitine levels between infants with and without BPD. We further mapped these significant AAs and carnitines together into two significant metabolic pathways. Among these, Gln and Met were the most notable metabolites involved. In the ROC analysis, we analyzed the results of seven significant metabolites at all of the four time points, and identified the Gln/C6:1 ratio at early total PN phase as a potential indicator of BPD, with a better sensitivity and specificity than single Gln at Point 2. Gln and C6:1, separately as substrate and intermediate products participating in the β-oxidation of mitochondria, may reflect the abnormal bioenergetics during early nutrition therapy in the pathogenesis of BPD indirectly. Now rare studies concerning metabolic indicators of BPD associated with early nutrition therapy were available.

Due to the complex pathophysiology of BPD, a single indicator for BPD is unlikely to be sufficient, and it would be essential to exploit indicators linked to specific clinical status allowing early recognition of neonates at risk for developing BPD. The metabolome is the full collection of the metabolites reflecting interactions between specific pathophysiological state and environmental stimuli, and makes it available to identify metabolite linked to specific pathogenesis. The metabolic pattern for BPD has been investigated in different biological liquids, as for amniotic fluid, cord blood, peripheral blood, tracheal aspirate (TA), bronchoalveolar lavage fluid (BALF) or in urine. Baraldi *et al.* performed metabolic analysis with amniotic fluid samples and found infants prone to develop BPD with higher levels of leucinic acid, hydroxy fatty acids and oxy fatty acids, and a reduced level of S-adenosyl methionine,[9] which may be associated with increased oxidative stress. La Frano *et al.* conducted a study on umbilical cord blood metabolomics and acquired an alteration in choline and choline-containing phospholipids, which may be responsible for an immaturity of lipid biosynthesis.[36] Piersigilli *et al.* evaluated TA samples from 68 neonates in the first week of life, and noted that five AAs including His, Glu, Cit, Gly and isoleucine, as well as two kinds of acylcarnitines C16-OH and C18:1-OH were higher in neonates with BPD, especially in preterm infants with GA below 27 weeks.[10] Furthermore, Fanos *et al.* and Pintus *et al.* analyzed urine metabolites collected from preterm infants with different GAs at first days after birth, respectively, then they obtained an inconsistent metabolic pattern.[37,38] As yet, no consensus of metabolic indicators was generally recognized for BPD, which may be caused by various biological liquids and specific clinical status, such as timing, maturity of infants born with different GAs. In our study, to evaluate metabolic analysis of infants with BPD during early nutrition transitional phase, we analyzed AAs and FAs pattern at four different nutritional time points dynamically. Although TA or BALF seemed to be lung specific to host response during the pathogenesis of BPD, they are not routinely acquired, especially in well babies without respiratory support. Hence, we collected DBSs from enrolled infants because of its easy availability and less invasion, besides they can reflect what is going on of the body during PN to EN supplement.

There are some limitations to our study. First, it had an observational design. The metabolism analysis was established with the significant AAs and carnitines with differences between the two groups. Our results revealed that seven in 60 metabolites detected here were capable of distinguishing infants prone to BPD; the role of the undetected intermediates mapped in the two major metabolic pathways identified remains to be proven. Due to the complex interaction between each individual metabolite and pathways in which they are involved, further biological studies are required. Second, the average GA and BW are much lower in infants with BPD than without; therefore, the metabolic patterns may be influenced by these differences. Considering the small sample size and higher risk of BPD in small gestational infants, we applied regression analysis to verify the correlation between significant metabolites and BPD, instead of roughly dividing infants into subgroups according to GA and BW, to avoid further differences in clinical status generated by further subgrouping infants. A prospective study involving a larger sample is necessary to confirm which metabolism indicators predict the occurrence of BPD in premature infants.

Conclusions

We applied metabolism analysis and identified seven significant AAs and carnitines capable of distinguishing BPD infants and identified the Gln/C6:1 ratio as a potential metabolic indicator of BPD. This work also demonstrates that the metabolic dysregulation of AA and carnitine profiles are involved in the development of BPD. Metabolism analysis may enhance the knowledge about the pathogenesis of BPD and provide a prospective therapy targeting for BPD in metabolomics.

AUTHORS' CONTRIBUTIONS

LW, Z-JH, W-HZ, and D-YL contributed to the design and review of the research. H-QS, D-YL, W-HZ, and LW conducted the investigation. H-QS, D-YL, LW, and W-HZ analyzed the data. LW wrote the article. Z-JH, W-YC and W-HZ reviewed the article. All authors endorsed the publication.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and publication of this article.

ETHICAL APPROVAL

The registry was conducted according to the Ethical Principles and Guidelines for the Protection of Human Subjects of Research set by Belmont Report, and approved by the medical ethics committee of Xinhua Hospital affiliated to Shanghai Jiao Tong University School of Medicine (XHEC-2016–139). The research was presented in such a manner as to assure preservation of the anonymity of the subjects.

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