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BASIC SCIENCE ARTICLE

Metabolomic profiling of intrauterine growth-restricted preterm infants: a matched case–control study

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BACKGROUND: The biochemical variations occurring in intrauterine growth restriction (IUGR), when a fetus is unable to achieve its genetically determined potential, are not fully understood. The aim of this study is to compare the urinary metabolomic profile between IUGR and non-IUGR very preterm infants to investigate the biochemical adaptations of neonates affected by early-onset-restricted intrauterine growth.
METHODS: Neonates born <32 weeks of gestation admitted to neonatal intensive care unit (NICU) were enrolled in this prospective matched case–control study. IUGR was diagnosed by an obstetric ultra-sonographer and all relevant clinical data during NICU stay were captured. For each subject, a urine sample was collected within 48 h of life and underwent untargeted metabolomic analysis using mass spectrometry ultra-performance liquid chromatography. Data were analyzed using multivariate and univariate statistical analyses.
RESULTS: Among 83 enrolled infants, 15 IUGR neonates were matched with 19 non-IUGR controls. Untargeted metabolomic revealed evident clustering of IUGR neonates versus controls showing derangements of pathways related to tryptophan and histidine metabolism and aminoacyl-tRNA and steroid hormones biosynthesis.
CONCLUSIONS: Neonates with IUGR showed a distinctive urinary metabolic profile at birth. Although results are preliminary, metabolomics is proving to be a promising tool to explore biochemical pathways involved in this disease.

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IMPACT:

- Very preterm infants with intrauterine growth restriction (IUGR) have a distinctive urinary metabolic profile at birth.
- Metabolism of glucocorticoids, sexual hormones biosynthesis, tryptophan-kynurenine, and methionine-cysteine pathways seem to operate differently in this sub-group of neonates.
- This is the first metabolomic study investigating adaptations exclusively in extremely and very preterm infants affected by early-onset IUGR.
- New knowledge on metabolic derangements in IUGR may pave the ways to further, more tailored research from a perspective of personalized medicine.

INTRODUCTION

The span time between conception and a child's second birthday—the first thousand days of life—is gathering an impressive and warranted scientific interest, since this is the moment when the health foundations of a lifetime are laid. The research on mechanisms and consequences of pregnancy complications and preterm birth assumes therefore huge relevance. Prematurity is a serious health problem and rates of births before 37 completed weeks of gestation have increased globally and now account for 11% of live births.[1] Because of the multiple adverse consequences associated with preterm birth, it has been proposed that it should be considered a chronic condition in itself, and that persons born prematurely require early evaluation, long-term follow-up, and preventive actions to reduce the risk of life-long consequences.[2] Among the causes of premature delivery, intrauterine growth restriction (IUGR) is a matter of great concern, since this condition may further endanger the clinical course and outcome of these babies. IUGR is indeed a paradigmatic condition in which a "hostile intrauterine environment" can impair fetal development so that the fetus is unable to achieve its genetically determined size.[3] The terms small for gestational age (SGA) and IUGR are often used interchangeably, nonetheless the latter is a prenatal diagnosis based on serial evaluations of a combination of ultrasound parameters including estimated fetal weight, abdominal circumference, and umbilical artery Doppler measurements.[4–6] Early-onset IUGR is clinically defined as that occurring at <32 weeks of

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gestation.[4] There is growing evidence that restricted intrauterine growth can predispose not only to immediate consequences at delivery and postnatal time, but also to several non-communicable diseases, including metabolic syndrome, obesity, coronary heart disease, hypertension, dyslipidemia, type 2 diabetes, and chronic lung and kidney diseases.[6,7] Conversely, SGA refers to a postnatal condition when a newborn's birth weight is below the 10th centile for gestational age, thus including those newborns who are simply constitutionally small, without being at risk of adverse outcomes.

The adaptation mechanisms occurring in the IUGR fetus are not easy to investigate. A limited number of studies have applied the metabolomic approach in efforts to characterize the biochemical adaptations associated with IUGR.[8–15]

Metabolomics is an evolving science that, through a closest-as-possible representation of a biological system's phenotype,[16–18] might provide a promising contribution to maternal and child health.[8–10,18–20] It can be applied to several biological samples through the targeted and untargeted approach. Targeted metabolomic aims at verifying a given pre-existing hypothesis by studying a fixed subset of certain metabolites, while untargeted approach is rather intended to generate a new hypothesis by measuring the totality of metabolites in a sample.[17,21] The goal is not the identification of each metabolite, but the discrimination of metabolic patterns associated with a given pathological condition since unexpected or unknown metabolites may turn out to be important in characterizing specific groups of subjects.[22]

In this pilot prospective case–control study, we applied the metabolite fingerprinting approach by means of mass spectrometry to verify if the urinary metabolome at birth is able to discriminate between IUGR and non-IUGR premature babies. As clearly stated by Pecks et al, it is of great clinical interest to obtain an unambiguous and distinctive metabolic profiling signature for the postnatal verification of the IUGR diagnosis.[23] Our selection of IUGR cases was therefore not based merely on birth weight centiles, but instead on a proper prenatal diagnosis of fetal growth restriction. To our knowledge, this is the first study addressing the issue specifically in extremely and very preterm neonates.

METHODS

Study population

This pilot prospective, matched, case–control study was conducted between January 2017 and September 2019 at the tertiary-level neonatal intensive care unit of the Women's and Children's Health Department of Padova, Italy. Extremely and very preterm neonates (born before 32 weeks of gestation) were eligible for the study excluding those with a major congenital or chromosomal abnormality, known or suspected congenital metabolic diseases, confirmed early-onset sepsis (positive blood or cerebrospinal fluid culture), and those who received transfusions (erythrocytes, plasma, or platelets), crystalloid solutions resuscitation or inotropes before the collection of urine samples. Early-onset IUGR was diagnosed by an experienced obstetric ultra-sonographer according to Hadlock fetal growth standards when estimated fetal weight or fetal abdominal circumference was below the 3rd centile or when estimated fetal weight or fetal abdominal circumference was below the 10th centile in association with an alteration of uterine or umbilical artery Doppler flow velocity profiles (pulsatility index above 95th centile).[5] For each eligible subject the following clinical data were collected: gestational age, birth weight, birth weight %ile (according to 2013 Fenton growth charts), sex, ethnicity, multiple pregnancy, antenatal steroids administration timing, type, and cause of premature delivery, arterial umbilical cord pH and base excess values, Apgar score at 5 min, respiratory support (need for surfactant and mechanical ventilation more than 24 h of life), hemodynamically significant patent ductus arteriosus, intraventricular hemorrhage (grade), C-reactive protein (CRP) increase. All included patients received the same nutritional regimen according to the internal protocol. A progressive total parenteral nutrition infusion was initiated at admission through a central umbilical or percutaneous line starting with 6–8 g/kg/day of glucose and 1.5 g/kg/day of amino acids with the target to achieve in the following days a non-protein energy >80 kcal/kg/day (glucose and lipids) and at least 3.5 g/kg/day of amino acids. Starting from birth until the second day of life patients received minimal enteral feeding 10–20 ml/kg/day with own's mother milk or donor human milk. Neonates enrolled in the IUGR group were a posteriori matched with the eligible neonates of similar clinical data that were not IUGR using the algorithm described in the Methods.

Ethical approval for the study was obtained from the Ethics Committee of Padua Hospital (protocol number: 4374/AO/17) and written informed consent was collected from all parents/guardians before the enrollment of the neonates in the study.

Sample collection and preparation

Urine sampling: at least 2 ml of urine were collected noninvasively within 48 h of birth, by placing a cotton ball inside the newborn' nappy and checking for the presence of urine every 30 min. The cotton ball was changed if the neonate did not urinate within 3 h of its placement or if it was contaminated with fecal material. After the neonate urinated, the cotton ball was placed in the barrel of a syringe and squeezed with the plunger to collect the absorbed urine in a container prewashed with MeOH, for metabolomic analysis. The same brands of nappies and cotton balls were used throughout the study. Samples were stored at −80 °C until analysis. The details of the preparation of the urine samples are reported in Supplementary Materials, Section S1.

Metabolomic analysis

The analysis was performed at the Mass Spectrometry and Metabolomics Laboratory of the University of Padua's and Women's and Children's Health Department (Città della Speranza Foundation, Pediatric Research Institute). Metabolic profiling was done in positive and negative ionization modes on an Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters, UK) coupled to a Quadrupole Time-of-Flight (QTof) Synapt G2 HDMS mass spectrometer (Waters MS Technologies, Ltd., Manchester, UK). The UPLC-HRMS conditions were optimized in terms of the peak shape, reproducibility, and retention times of the analytes. Chromatography was performed using an Acquity HSS T3 (1.7 µm, 2.1 × 100 mm) and an Acquity HILIC (1.7 µm, 2.1 × 100 mm) column (Waters Corporation, Milford, USA), at 50 and 40 °C, respectively. For mass accuracy, a LockSpray interface was used with a 20 µg/l leucine enkephalin. Data were collected in continuous mode in a scanning range of 20–1200 m/z, with lockmass scans collected every 10 s, averaging over 3 scans for mass correction purposes. In the analytical sequences, quality control samples (QC) and standards solution samples (Mix) were used to assess reproducibility and accuracy during the analysis, and, together with Baseline samples (Blank), to examine the metabolite content of the urine samples. Samples preparation and characteristics are reported in Supplementary Materials, Section S1. An example of a chromatographic profile of a QC sample is reported in Fig. 1.

Data were pre-processed using Progenesis software (Waters Corporation, Milford, USA). The ion intensities for each peak detected were quantified as peak area and normalized on the basis of the calibration models obtained for the QCs. Thus, probabilistic quotient normalization was applied to remove dilution effects on the sample concentrations, and variables with a coefficient of variation greater than 20% in the QCs were excluded. The data tables generated were submitted for statistical analysis.

Matching IUGR and non-IUGR neonates

The procedure implemented to extract the two groups of neonates, IUGR (cases) and non-IUGR (controls), from the eligible neonates is described in the following. The distance matrix between the neonates of the IUGR group and the eligible non-IUGR neonates was calculated considering all the demographic and perinatal data recorded excluding birth weight and birth weight %ile. For each IUGR neonate, the non-IUGR neonate with the minimum distance was selected and the pairs of case–control obtained were sorted on the basis of the increasing distance. The pairs with the greatest distance were iteratively excluded until the set of the selected IUGR neonates and the set of the selected controls showed p values of the t-test or of the Mann–Whitney test greater than 0.05 in the case of numerical data normally or non-normally distributed, respectively, and p value of the Pearson χ^2 test greater than 0.05 in the case of categorical data. More details about the distance used in the algorithm are reported in Supplementary Materials, Section S2.

Statistical data analysis

The demographic and perinatal characteristics of the recruited neonates were investigated by applying a t-test for normally distributed data, a

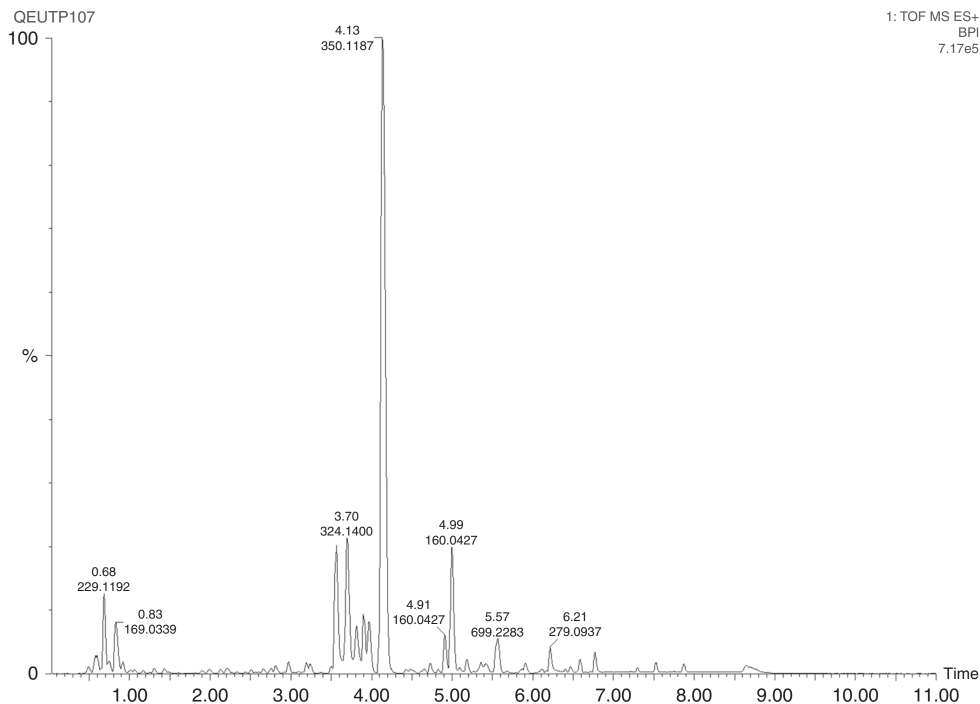

Fig. 1 Chromatographic profile of the pool of the analyzed urine samples. A BPI UPLC-MS chromatogram profile of a urine pool sample (QC) run in positive ESI mode. ESI Electrospray Ionisation, BPI Base Peaks Intensity.

Mann–Whitney test for non-normally distributed data, and a Pearson χ^2 test for categorical data, considering statistically significant tests with p values less than 0.05. Normality was assessed using the Shapiro–Wilk test assuming normally distributed data for p value > 0.10.

Both univariate and multivariate statistical data analyses were applied to characterize the two groups of newborns on the basis of metabolomic fingerprinting. Firstly, exploratory data analysis by means of principal component analysis (PCA) was performed to identify outliers. Then, the Mann–Whitney test controlling the false discovery rate by the Storey method[24] was applied to compare the median of each variable in the two groups investigated. We assumed q value < 0.10 as a level for assessing significant features. Multivariate data analysis was based on projection to latent structures regression-discriminant analysis (PLS-DA) with stability selection.[25,26] Class membership was assessed by applying linear discriminant analysis to the scores of the PLS-DA model built by autoscaling the dummy variables specifying the class of the sample. Matthew correlation coefficient (MCC) has been used to measure the goodness-of-fit of the model. In the stability selection procedure, variable influence on projection score was used for ranking the predictors within the feature selection step. A total of 500 subsets were extracted by bootstrap and relevant features were identified assuming a significance level equal to 0.05. Five-fold cross-validation was applied in model optimization and the related $MCC_{5\text{-fold}}$ was calculated. Moreover, a permutation test on the class response (1000 random permutations) was performed to highlight over-fitting. Data were mean-centered prior to performing data analysis.

The results of the multivariate data analysis were merged with those of the univariate data analysis to obtain a set of relevant variables, which was submitted to the annotation step.

Over-representation pathway analysis on the set of annotated features was performed considering 80 pathways for Homo sapiens.

Data pre-processing and analyses were conducted using in-house R-functions implemented on the R 3.6.0 platform (R Foundation for Statistical Computing, Vienna, Austria). Over-representation pathway analysis was performed by MetaboAnalyst 3.0.

Metabolite annotation

The relevant variables highlighted by data analysis were annotated by searching the Human Metabolome Database, the METLIN metabolite database, and our in-house database. In addition, urine samples with a higher ion intensity were injected in MS/MS mode to obtain more information on the ions' structure by studying the relative fragments. If the commercial standards were already available at our laboratory, the mass-to-charge ratio (m/z), retention time (Rt), and ion fragments in the urine were compared directly with the corresponding standard. These procedures enabled us to classify the selected features with different levels of annotation.[27]

RESULTS

Demographic and perinatal data

Among 160 infants eligible at birth, 77 were excluded because of missing parental consent, congenital disease, haemodynamic instability, confirmed early-onset sepsis, need for blood component transfusions, or inadequate urine sample collection. A total of 83 neonates were enrolled, of whom 15 were diagnosed prenatally with early-onset IUGR and were matched with 19 non-IUGR controls. The demographic and perinatal characteristics of the 34 selected neonates are reported in Table 1. The two groups were comparable in regard to gestational age, sex, ethnicity, multiple pregnancy, mode of delivery, timing of administration of betamethasone during pregnancy, signs of fetal/neonatal distress (abnormal cardiotocography, Apgar score at 5 min of life, umbilical cord arterial pH, umbilical cord arterial base excess), need for surfactant administration and for mechanical ventilation (>24 h of life), presence of patent ductus arteriosus, intraventricular hemorrhage and increased CRP levels (>10 mg/l). As expected, the IUGR group had a median birth weight and a median birth weight %ile significantly lower than non-IUGR controls. An over-representation of females was observed in the non-IUGR group, even if the difference with respect to the IUGR group was not significant (p value = 0.08). Since the urinary metabolic profile may be influenced by sex, we excluded bias by exploring the latent space of the PLS-DA models and evaluating the effect of sex directly on the metabolic profile.

For every newborn enrolled in the IUGR group the criteria for diagnosis, the indication for delivery, and the arterial umbilical cord pH and base excess values are reported in Table 2. Premature birth of infants diagnosed with IUGR was due both to maternal (preeclampsia, refractory hypertension, HELLP syndrome) and fetal

Table 1. Demographic and perinatal characteristics of the recruited neonates.

	IUGR ($n = 15$)	Non-IUGR ($n = 19$)	p value
Gestational age [days]	211 (11)	207 (10)	0.353
Birth weight [g]	1029 (239)	1302 (198)	<0.001
Birth weight [%ile]	12 [9]	48 [39]	<0.001
Sex (females)	50% (7)	79% (15)	0.08
Ethnicity			0.44
Caucasian	93% (14)	100% (19)	
African or Hispanic	0% (0)	0% (0)	
Asiatic or Hispanic	7% (1)	0% (0)	
Multiple pregnancies	20% (3)	26% (5)	>0.99
Mode of delivery (CS)	100% (15)	89% (17)	0.49
Antenatal steroids administration	100% (15)	95% (18)	>0.99
Timing of administration (days before delivery)	4 [5]	6 [10]	0.68
Signs of fetal/neonatal distress			
Abnormal cardiotocography	27% (4)	21% (4)	0.71
Apgar score at 5 min	8 [0]	8 [0.5]	0.49
Umbilical cord arterial pH	7.27 [0.07]	7.30 [0.12]	0.23
Umbilical cord arterial BE	−2.8 [6]	−3.9 [4.8]	0.16
Severe neonatal acidemia (umbilical cord arterial pH < 7.0)	0% (0)	5% (1)	>0.99
Surfactant	40% (6)	74% (14)	0.08
Mechanical ventilation (>24 h)	20% (3)	37% (7)	0.45
PDA	33% (5)	37% (7)	>0.99
IVH	13% (2)	0% (0)	0.19
CRP > 10 mg/l	20% (3)	16% (3)	>0.99

Numerical data normally distributed are reported as mean (standard deviation) whereas non-normally distributed data as median [interquartile range], and categorical data as prevalence (absolute number).
CS cesarean section, *BE* base excess, *PDA* patent ductus arteriosus, *IVH* intraventricular hemorrhage, *CRP* C-reactive protein.

indication (abnormal Doppler velocimetry pattern, abnormal cardiotocography). Babies born because of fetal indications did not show worse umbilical cord acid-base balance values than those born because of maternal causes (p value = 0.60), indicating that none of IUGR patients had to face severe decompensation.

Among the non-IUGR infants, the causes of premature birth were premature labor with or without premature rupture of membranes, chorioamnionitis, placenta abruption or other placental problems, and complications to the co-twin in multiple pregnancies.

Untargeted metabolomic profiling

The data pre-processing of the untargeted metabolomic profiling of the urine samples generated four datasets, two for the negative ionization mode called T3 NEG (composed of 599 time@mass variables) and HILIC NEG (with 844 time@mass variables) being obtained from the raw data produced by means of the Acquity HSS T3 and the Acquity HILIC columns, respectively, and two for the positive ionization mode indicated as T3 POS (composed of 1080 time@mass variables) and HILIC POS (with 957 time@mass variables) being obtained by Acquity HSS T3 and the Acquity HILIC columns, respectively. No outliers were detected by PCA on the basis of the T2 and Q tests assuming a significance level of 0.05.

Univariate data analysis highlighted 4 variables in the T3 NEG data set, 8 variables in the HILIC NEG data set, 12 in the T3 POS data set, and 6 in the HILIC POS data set as relevant, for a total of 30 features relevant to discriminate the IUGR group and the controls.

Multivariate data analysis based on PLS-DA with stability selection discovered 271 variables as relevant. Specifically, a PLS-DA model with 2 latent variables, MCC = 0.80 and $MCC_{5\text{-fold}} = 0.52$ was obtained for the T3 NEG data set (the score scatter plot of the model is reported in Supplementary Fig. S1 as an example), and 51 variables were selected as relevant by stability selection. For the HILIC NEG data set, the model showed 2 latent variables, MCC = 0.75, $MCC_{5\text{-fold}} = 0.54$ and 84 variables were selected. For the T3 POS data set, the PLS-DA model presented 2 latent variables, MCC = 0.62 and $MCC_{5\text{-fold}} = 0.46$, and 75 variables were selected as relevant. For the HILIC POS data set, the PLS-DA model showed 2 latent variables, MCC = 0.51, $MCC_{5\text{-fold}} = 0.32$ and 61 variables resulted to be relevant.

The 30 features discovered by univariate data analysis were merged with the 271 variables obtained by multivariate data analysis obtaining a set of 286 relevant features that were submitted to data annotation. We were able to annotate 57 variables. As reported in Table 3, 14 variables were annotated at level 1 ("identified metabolites"), 6 at level 2 ("putatively annotated compounds") and 37 at level 3 ("putatively characterized compound classes").[27] Over-representation pathway analysis conducted on the annotated variables at levels 1 and 2 revealed four perturbed pathways (p value < 0.02). Specifically, pathways related to tryptophan metabolism, aminoacyl-tRNA biosynthesis, histidine metabolism, and steroid hormone biosynthesis were perturbed. The results of over-representation pathway analysis are reported in Fig. 2.

DISCUSSION

IUGR is a paradigmatic condition, in which an impaired intrauterine environment might hamper infant development and predispose to several major diseases later in life,[6,7] though the exact mechanisms are mostly uncovered as well as an early biomarker capable to assess the severity of the disease.

The aim of our study was to compare the urinary metabolic profile of preterm neonates affected by early-onset IUGR and non-

Table 2. For every newborn enrolled in the IUGR group, the criteria for diagnosis, the indication for delivery, and the arterial umbilical cord pH, and base excess (BE) values are reported.

	Criteria met for IUGR diagnosis	Indication for delivery	Arterial umbilical cord pH	Arterial umbilical cord BE
1	EFW + AC <3° %ile	Preeclampsia	7.12	−8.2
2	AC <10° %ile + PIUA >95° %ile	Preeclampsia	7.10	−5.6
3	AC <10° %ile + PIUA + PIUtA >95° %ile	Abnormal Doppler velocimetry pattern	7.3	−2.8
4	AC <3° %ile, PIUA >95° %ile	Abnormal Doppler velocimetry pattern + abnormal cardiotocography	7.22	−1.6
5	EFW <3° %ile	Preeclampsia	7.33	0.9
6	AC <10° %ile + PIUA >95° %ile	Abnormal Doppler velocimetry pattern	7.24	4.1
7	AC <10° %ile + absent end diastolic flow in UA	Abnormal Doppler velocimetry pattern	7.25	−6.3
8	AC <10° %ile + PIUA >95° %ile	HELLP syndrome	7.27	0.4
9	AC <10° %ile + PIUA >95° %ile	Refractory hypertension + abnormal cardiotocography	7.32	2.5
10	AC <10° %ile + PIUA and PIUtA >95° %ile	Refractory hypertension + abnormal cardiotocography	7.33	−2.5
11	AC <10° %ile + PIUA and PIUtA >95° %ile	Refractory hypertension	7.27	−2.7
12	EFW and AC <3° %le	Preeclampsia	7.31	−4.2
13	AC <10° %ile + PIUA >95° %ile	Preeclampsia	7.25	−3
14	EFW and AC <3° %le	Abnormal Doppler velocimetry pattern	7.26	−2.8
15	AC <3° %ile, PIUA >95° %ile	Abnormal Doppler velocimetry pattern	7.31	−5.7

EFW estimated fetal weight, *AC* abdominal circumference, *PIUA* pulsatility index in the umbilical artery, *PIUtA* pulsatility index in the uterine artery, *HELLP* hemolysis, elevated liver enzymes, and low platelets.

affected matched controls. The metabolite fingerprinting through UPLC-HRMS of early urine samples revealed an evident clustering of subjects (IUGR versus non-IUGR infants). The annotation of the variables that best discriminate between the two groups has highlighted interesting putative metabolic pathways perturbations that might be involved in this condition.

Tryptophan pathway

Tryptophan resulted to be higher in IUGR neonates when compared to non-IUGR. In addition, within the metabolic pathway of tryptophan, other five variables were able to discriminate between the two groups, which were annotated as 3-indoleproprionic acid, 5-hydroxyindoleacetic acid, 5-hydroxykynurenamine, indolelactic acid, and 3-hydroxyanthranilic (Fig. 3).

Tryptophan is an essential amino acid that can be metabolized into a variety of small biologically active compounds, each capable of influencing a large number of metabolic pathways. Tryptophan metabolism can be divided into two main pathways: the prevalent one (>90% of the whole tryptophan biotransformation), namely the "kynurenine shunt", in which the indole ring is broken to produce cell-response compounds as kynurenines, nicotinic acid, and the nicotinamide adenine dinucleotide (NAD+), and the limited one (approximately 10% of the whole tryptophan transformations), in which the indole ring is retained and tryptophan is transformed in neurotransmitters/hormones as serotonin, N-acetyl-serotonin, melatonin and trace amines as tryptamine and derivatives.[28] During pregnancy tryptophan is actively transported to the fetus via the placenta where it plays an important role in protein synthesis for growth and development.[29,30] Consistently with our findings, previous research has shown increased tryptophan cord blood concentrations in IUGR infants[11,31,32] while other studies have reported a reduction of this amino acid.[33,34] Hernandez-Rodriguez et al. demonstrated that binding kinetics between tryptophan and albumin might be altered in infants with IUGR when compared to controls, with a minor affinity and binding capacity and therefore higher level of free L-tryptophan in plasma.[32] Interestingly Murthi et al. showed that the expression of the enzymes 2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO), which are expressed in the human placenta and are responsible for the oxidation of tryptophan into kynurenine and its downstream metabolites, was lower in placentas obtained by IUGR pregnancies. In addition, an impaired oxygen environment significantly reduced the expression of all the major metabolites and enzymes involved in kynurenine pathway.[35] Our findings further support the hypothesis that kynurenine shunt might be less active in IUGR.

Except for a reduction in levels of 5-hydroxyindoleacetic, a serotonin metabolite, the tryptophan-serotonin-melatonin pathway seems not to be affected by IUGR in our sample of premature babies.

Methionine-cysteine metabolism

According to our preliminary results, methionine levels were lower in IUGR neonates whereas cysteine levels were higher in IUGR neonates. Previous metabolomic-based studies have shown similar perturbations.[36] Nonetheless results were controversial with some studies showing a higher concentration of methionine in IUGR when compared with controls while others lower levels.[11,37,38] According to Horgan et al., methionine was upregulated in placental villous explants from SGA newborns when exposed to oxygen deprivation.[39] Also, Favretto et al. found an elevation of methionine in cord blood collected by IUGR neonates.[31] In the study performed by Wang et al. methionine-cysteine pathway resulted to be one of the most disrupted pathways in IUGR twins.[37] Methionine is an essential amino acid, whose metabolism is composed of two main pathways: the transmethylation cycle and the trans-sulfuration pathway. The transmethylation cycle consists of the conversion of methionine and ATP into s-adenosylmethionine (SAM).[40] SAM is ubiquitous and is the main methyl-donor for numerous reactions involved in DNA synthesis and regulation of gene expression.[41] The possible

Table 3. Annotated variables.

	m/z	Type	FC	Level	HMDB ID	Annotation
1	145.053	>IUGR	2.34	1	HMDB02302	3-Indolepropionic acid
2	203.083	>IUGR	2.10	1	HMDB0000929	L-Tryptophan
3	154.051	>Non-IUGR	0.28	1	HMDB0001476	3-Hydroxyanthranilic acid
4	110.072	>IUGR	1.24	1	HMDB00177	L-Histidine
5	122.028	>IUGR	1.75	1	HMDB00574	L-Cysteine
6	316.115	>Non-IUGR	0.58	1	HMDB00489	Aspartylglycosamine
7	227.115	>Non-IUGR	0.25	1	HMDB00033	Carnosine
8	267.075	>Non-IUGR	0.39	1	HMDB00195	Inosine
9	407.208	>Non-IUGR	0.50	1	HMDB0006758	Dihydrocortisone
10	148.045	>Non-IUGR	0.88	1	HMDB00696	L-Methionine
11	347.222	>Non-IUGR	0.54	1	HMDB0003259	5Beta-dihydrocortisol
12	371.259	>Non-IUGR	0.13	1	HMDB00391	7-ketodeoxycholic acid
13	287.201	>IUGR	2.75	1	HMDB0000053	Androstenedione
14	305.211	>IUGR	5.66	1	HMDB0004611	7alfa-hydroxydehydroepiandrosterone (7a-OH-DHEA)
15	168.063	>IUGR	1.43	2	HMDB00808	N-Butyrylglycine
16	192.048	>Non-IUGR	0.57	2	HMDB00763	5-Hydroxyindoleacetic acid
17	279.134	>Non-IUGR	0.80	2	HMDB03426	Pantetheine
18	174.124	>Non-IUGR	0.53	2	HMDB00856	N-a-Acetylcitrulline
19	274.092	>Non-IUGR	0.76	2	HMDB00671	Indolelactic acid
20	227.103	>Non-IUGR	0.66	2	HMDB04076	5-Hydroxykynurenamine
21	102.057	>IUGR	1.73	3	HMDB0000452	L-2-aminobutyric acid
22	231.088	>IUGR	1.27	3	HMDB02091	Isovalerylglucuronide
23	246.045	>IUGR	1.52	3	HMDB02381	N-Acetylcystathionine
24	137.026	>IUGR	1.75	3	HMDB03503	3-(3,4-Dihydroxyphenyl)lactic acid
25	195.065	>IUGR	1.43	3	HMDB02237	3,4-Dimethylbenzoic acid
26	615.223	>IUGR	36.93	3	HMDB06581	3-Sialyl-N-acetyllactosamine
27	284.08	>IUGR	1.51	3	HMDB04985 HMDB02248	Aspartylysine Gamma glutamyl ornithine
28	111.044	>IUGR	1.59	3	HMDB02074	2,2-Dimethylsuccinic acid
29	271.167	>IUGR	1.75	3	HMDB00145	Estrone
30	306.063	>IUGR	1.62	3	HMDB00229	Nicotinamide ribotide
31	201.113	>IUGR	1.57	3	HMDB00350	3-Hydroxysebacic acid
32	127.039	>IUGR	1.24	3	HMDB00393 HMDB00522	3-Hexenedioic acid 3-Methylglutaconic acid
33	328.045	>IUGR	1.82	3	HMDB01314 HMDB01397	Cyclic GMP GMP
34	103.05	>IUGR	1.49	3	HMDB01366	Purine
35	115.004	>Non-IUGR	0.68	3	HMDB00044 HMDB00134	Ascorbic acid Fumaric acid
36	130.051	>Non-IUGR	0.78	3	HMDB06272	5-Amino-2-oxopentanoate
37	77.0399	>Non-IUGR	0.64	3	HMDB00873 HMDB04284	4-Methylcatechol Tyrosol
38	277.12	>Non-IUGR	0.80	3	HMDB03426	Pantetheine
39	257.094	>Non-IUGR	0.24	3	HMDB00594	Glutamylphenylalanine
40	174.124	>Non-IUGR	0.53	3	HMDB00856	N-a-Acetylcitrulline
41	247.13	>Non-IUGR	0.59	3	HMDB00819 HMDB02832	Normetanephrine Methylnoradrenaline
42	116.107	>Non-IUGR	0.54	3	HMDB00339	2-Methylbutyrylglycine
43	250.094	>Non-IUGR	0.56	3	HMDB00230	N-Acetylneuraminic acid
44	257.079	>Non-IUGR	0.81	3	HMDB04629	O-Desmethylangolensin
45	296.101	>Non-IUGR	0.91	3	HMDB00859	1-Methylguanosine
46	149.048	>Non-IUGR	0.91	3	HMDB00127 HMDB00859 HMDB04308	D-Glucuronic acid Methylhippuric acid 7,9-Dimethyluric acid

Table 3. continued

	m/z	Type	FC	Level	HMDB ID	Annotation
47	142.052	>Non-IUGR	0.38	3	HMDB01138	N-Acetylglutamic acid
48	259.131	>Non-IUGR	0.54	3	HMDB03581	Dethiobiotin
49	305.096	>Non-IUGR	0.63	3	HMDB01067	N-Acetylaspartylglutamic acid
50	119.05	>Non-IUGR	0.53	3	HMDB02340	2-Methylbenzoic acid
51	331.227	>Non-IUGR	0.53	3	HMDB00016	Deoxycorticosterone
52	463.199	>Non-IUGR	0.47	3	HMDB00313	16b-Hydroxyestrone
53	225.087	>Non-IUGR	0.61	3	HMDB00732 HMDB00830 HMDB00877	Hydroxykynurenine Neuraminic acid Umanopterin
54	248.114	>Non-IUGR	0.66	3	HMDB03333	8-Hydroxy-deoxyguanosine
55	809.701	>Non-IUGR	0.59	3	HMDB04976 HMDB05435	Glucosylceramide (d18:1/26:1(17Z) TG(16:1(9Z)/16:1(9Z)/18:2(9Z,12Z))[iso3]
56	231.098	>Non-IUGR	0.71	3	HMDB00497 HMDB02335	5,6-Dihydrouridine Aspartyl-L-proline
57	169.134	>Non-IUGR	0.54	3	HMDB03581	Dethiobiotin

Type: >IUGR indicates that the median was higher in the IUGR group than in the non-IUGR group; >non-IUGR indicates that the median was higher in the non-IUGR group than in the IUGR group. FC: fold change measured as ratio between median in the IUGR group and median in the non-IUGR group. Level: annotation level according to reference.[25] *HMDB ID*, identifier used in the Human Metabolome Database.

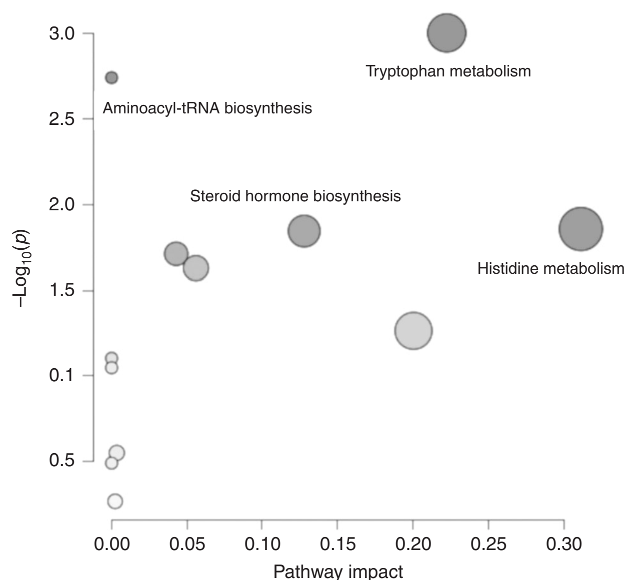

Fig. 2 Over-representation pathway analysis. The impact of each perturbed pathway is shown against its negative log *p* value ($-\log_{10}(p)$); in the figure are indicated the names of the perturbed pathways with *p* value < 0.02.

association between methionine availability and epigenetic changes during fetal and postnatal life in IUGR infants is worth further investigation, also in the light of its possible implication in "fetal-neonatal programming".

Histidine metabolism

Our analysis revealed a possible perturbation in mutual conversion between histidine and carnosine, with a higher level of the first and lower level of the latter in IUGR neonates. Histidine concentration was found to be higher in cord blood of IUGR subjects in some previous metabolomic studies[31] and lower in others.[42] Histidine is another essential amino acid involved in several metabolic pathways. 3-Methylhistidine is one of the main derivatives of histidine, formed by the post-translational methylation of histidine residues of actin and myosin, and has been used as a marker of skeletal muscle protein catabolism.[43] Notably, in a group of preterm babies receiving dexamethasone for bronchopulmonary dysplasia, a substantial increase in 3-methylhistidine excretion as a possible sign of increased muscular catabolism, was found after steroid treatment.[44] Although our study did not show an increase in urinary 3-methylhistidine, it could be speculated that increased histidine excretion might be attributable to a catabolic status in IUGR subjects. Carnosine is another intriguing metabolite that was able to discriminate between the two groups, being present in lower levels in IUGR infants. Carnosine is produced through hydrolysis of ATP from histidine and beta-alanine and is mainly present in skeletal muscle where it plays an important role as intracellular pH buffer, heavy metal chelator, and anti-glycating agent. Experimental studies on animals suggest that carnosine may play a role as antiaging, probably through its antioxidant properties.[45] Oxidative stress is known to play a key role in IUGR pathogenesis and complications; indeed, placental insufficiency and chronic intrauterine hypoxia lead to the generation of free radicals that fetal antioxidant systems are not able to neutralize.[45,46] Interestingly, there is growing evidence from in vitro and in vivo experimental studies that carnosine administration could provide antioxidative protection by reducing the level of oxidative damage markers, particularly of malondialdehyde[47] but also of isoprostanes.[48] It might therefore be speculated that carnosine is somehow consumed due to its activity as an oxidative product scavenger in the IUGR group.

The above-mentioned amino acids (tryptophan, methionine, cysteine, and histidine) are all exogenously provided to premature babies through parenteral nutrition starting from the first hours of life, thus potentially influencing the urinary metabolome. All included patients received the same nutritional regimen according to the internal protocol, so that it is unlikely that the emerging difference between the two groups is imputable to an external supply. Nonetheless, if the perturbation of these metabolic pathways is confirmed and persistent over time, this is probably proof that an individualized nutritional approach should be fostered to meet the specific requirements of this special category of newborns.

Steroid hormone biosynthesis

Steroid hormone biosynthesis perturbation emerged from the metabolomic profile comparison between IUGR and non-IUGR

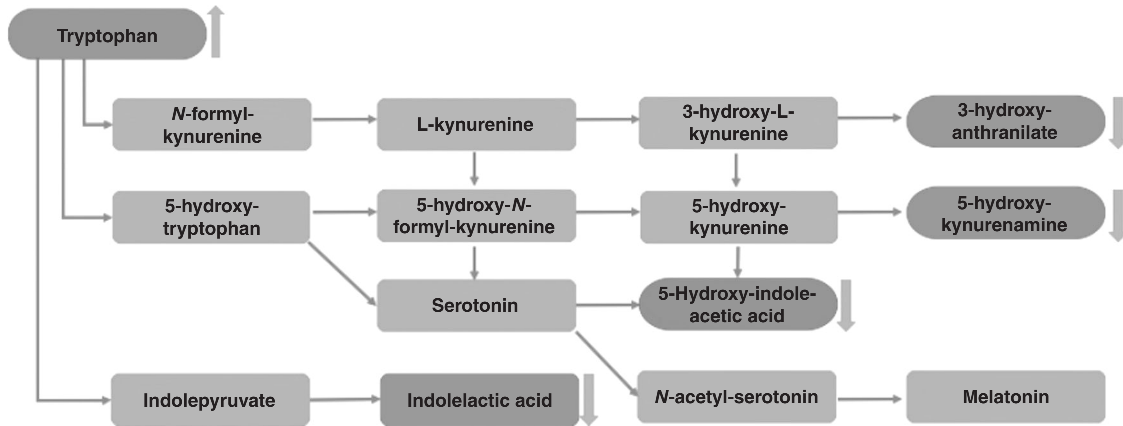

Fig. 3 Scheme representing the metabolites involved in the tryptophan metabolism. In red are indicated the annotated metabolites (levels 1 and 2). Upward arrows indicate metabolites higher in the IUGR group, whereas downward arrows metabolites lower in the IUGR group.

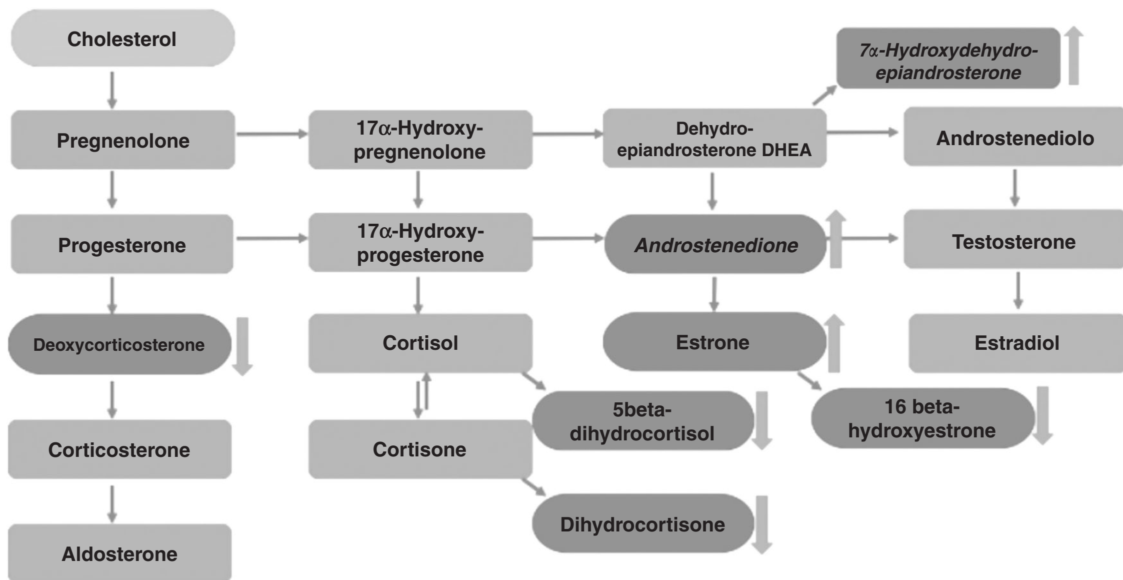

Fig. 4 Scheme representing the metabolites involved in the steroid hormone biosynthesis. In red are indicated the annotated metabolites (levels 1–3). Upward arrows indicate metabolites higher in the IUGR group, whereas downward arrows metabolites lower in the IUGR group.

subjects. In particular, seven metabolites were found to be differently distributed in the two groups: dihydrocortisol, dihydrocortisone, deoxycorticosterone, and 16beta-hydroxysterone were lower in IUGR, while estrone, androstenedione, and 7alfa-hydroxydehydroepiandrosterone (7α-hydroxy-DHEA) were higher (Fig. 4).

During pregnancy, placenta synthesizes the corticotropin–releasing hormone (placental CRH), which stimulates the secretion of adrenocorticotropic hormone (ACTH) by the anterior pituitary gland and, consequently, the synthesis of cortisol, glucocorticoids, mineralocorticoids, and dehydroepiandrosterone (DHEA).[49] Recent evidence shows that an elevation of placental CRH is involved in preterm birth and in pregnancies complicated by hypertension and IUGR.[50,51] Glucocorticoids play a pivotal role in fetal development: appropriate exposure to glucocorticoids during gestation is requested for the maturation of fetal organs, particularly the respiratory system. Despite these positive effects, an over-exposure of the fetus to glucocorticoids not only causes fetal growth restriction but also predisposes it to the development of chronic diseases such as metabolic syndrome.[52] It is believed that prenatal exposure to undernutrition and hypoxia in IUGR prompts the activation of the hypothalamus–pituitary–adrenal axis as an adaptive response to stress.[51] Nonetheless, the relation between cortisol and IUGR has been sometimes contradictory, with some analyses revealing higher cortisol and lower ACTH levels in cases of IUGR[53] and others showing the opposite.[54] Interestingly, our study showed that the two main downstream metabolites of both cortisol and cortisone, dihydrocortisol and dihydrocortisone, respectively, were reduced in IUGR. Those two metabolites are the results of reduction reactions performed by reductase. It is believed that the balance between the parent steroids (cortisol and cortisone) and their inactive metabolites could be crucial for the modulation of metabolic and anti-inflammatory actions of glucocorticoids.[55] In addition, 7α-hydroxy-DHEA, a derivative of DHEA, was increased in IUGR neonates. Noteworthy, 7α-hydroxy-DHEA, has been reported to be a strong inhibitor of 11beta-hydroxysteroid dehydrogenase (11β-HSD) type 1, an enzyme that catalyzes the conversion of inactive cortisone to active cortisol.[56] The increase of this metabolite in the urine of IUGR neonates might represent a compensatory mechanism to reduce circulating cortisol through the inhibition of 11β-HSD type 1. These results could suggest the presence of a disturbed glucocorticoid pattern in the growing IUGR fetus with possible detrimental effects. We cannot exclude that antenatal corticosteroid administration may have influenced the metabolomic

profiles of both our groups but in what way and degree we are not able to state. Nonetheless, given the high percentage of risk-pregnancy in which steroids are administered this is probably an unavoidable bias when doing research on premature infants. Dedicated research, perhaps by means of metabolomics, should address specifically this issue. Other downstream metabolites of DHEA, belonging to the sex hormones pathway, emerged to be higher in IUGR babies: androstenedione, estrone, and 16beta-hydroxyestrone. A recent study has compared the sex steroid hormones profile in cord blood collected from SGA and adequate for gestational age preterm infants. Only females born SGA had significantly higher levels of androstenedione, while males born SGA showed decreased levels of estrone compared to controls.[57] Although these results are only partly concordant with ours, they prompt further investigations. Sex may probably influence the urinary metabolomics profile in infants, in particular affecting fatty acids, apolipoprotein, and cholesterol levels, which were found to be higher in female newborn cord blood than in males in a study performed by Mansell et al. based on nuclear magnetic resonance metabolomic analysis.[58] Notably, another analysis of the urinary metabolic profiles of 90 newborns followed up from birth to 4 months of age revealed metabolites significantly associated with age, weight, and height, but not with sex.[59] In our study, we were not able to discover the effect of sex on the collected urine samples. Nonetheless, the latent structures discovered by PLS-DA were not influenced by sex.

The present study revealed an evident clustering of metabolome features of urine collected from subjects IUGR when compared with non-IUGR controls. Metabolomics turns out to be a useful tool to explore and better understand the pathophysiological alterations of IUGR. Our findings, thought preliminary, might pave the ways to further investigations focused on tryptophan-kynurenine shunt, methionine-cysteine and histidine-carnosine pathways, and glucocorticoids/sexual hormones metabolism in IUGR.

The value of our work is the robust methodology starting from the choice of UPLC-HRMS as an analytical method, through advanced statistical analysis, to a rigorous annotation process and, as a major point, the exclusion of possible confounding factors, such as blood components transfusions, the latter being rarely taken into account in previous metabolomics studies. Another strength of this study is the comparability of the two groups in terms of gestational age and main postnatal comorbidities, thus allowing us to state that the observed differences are probably due right to the condition of IUGR. Furthermore, the case group was not selected through the criterion of SGA definition, in other terms to enroll merely those babies with a birth weight <10th %ile for gestational age, but through the criterion of a thorough early IUGR diagnose, performed indeed prenatally by experienced obstetric ultra-sonographers. Nonetheless, the authors recognize that, despite the matching procedure has been developed to reduce possible confounders, subjects belonging to the non-IUGR control sample are, by definition, "not healthy" because born preterm, thus their metabolome might be influenced by other factors. This is an ineliminable and inevitable bias though: whether we had considered healthy term infants as controls, the issue of prematurity would have played an even higher confounding role. Indeed, data published by Ernst et al. in a cohort of almost 300 newborns with gestational ages from 28 to 42 weeks clearly displayed a large variation in the neonatal blood metabolome composition related to different gestational ages.[60] Another limitation of the study is the small sample size that prompts us to be cautious with the generalization of results. Nevertheless, previous studies based on the metabolomics approach for the evaluation of IUGR infants could rely on a similar number of subjects. Then the authors recognize that metabolomic signatures distinctive of the sickest IUGR neonates may have been missed as a result of the stringent patient selection criteria, above all the exclusion of infants with hemodynamic instability or requiring blood components transfusions. Moreover, although a stratification of IUGR subjects based on the severity of Doppler velocimetry abnormalities goes beyond the aims of this pilot study, it would definitely be worth being investigated in the future. Further investigations, including a larger cohort and a targeted metabolomic analysis on plasma samples, are ongoing in order to overcome these limitations and possibly confirm these results.

CONCLUSIONS

Urine metabolomic profiling of the cohort enrolled in this pilot study showed a clear clustering between IUGR and non-IUGR infants. Notably, we further support the hypothesis of derangement in glucocorticoids metabolism, sexual hormones biosynthesis, tryptophan-kynurenine shunt and methionine-cysteine pathways, and a possible disruption in the antioxidant system (histidine-carnosine bio-transformations). These findings, though preliminary, could suggest an altered metabolic profile in IUGR patients and deserve more thorough investigations including a long-term follow-up.

Hopefully in the future, new insights from metabolomic science could support neonatologists to improve the quality and appropriateness of IUGR infants' care, by individualizing the nutritional supply and therapies of this special category of newborns.

DATA AVAILABILITY

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

E.P. contributed to study conception and design, acquisition, and interpretation of data and drafted the article. G.V. and E.B. contributed to study conception and design, interpretation of data, and critically reviewed the manuscript. M.S., G.G., and P.P. contributed to study conception and design, analysis, and interpretation of data, and critically reviewed the manuscript. L.B., S.V., and L.M. critically reviewed the manuscript. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

COMPETING INTERESTS

The authors declare no competing interests.

CONSENT TO PARTICIPATE

A written informed consent was collected from all parents/guardians before the enrollment of the neonates in the study.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41390-022-02292-5.

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