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Original article

Urinary metabolomics in term newborns delivered spontaneously or with cesarean section: preliminary data

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Abstract

Introduction: In the last years the uncritical attitude towards cesarean section (CS) has been associated with the fast emergence of 'modern' diseases such as early pediatric obesity, asthma, type 2 diabetes mellitus and dermatitis. Increasing evidence shows that babies born at term by vaginal delivery (VD) have a different physiology at birth, with subsequent influence on adult health. In relation to these short-term physiological changes, in the present study we aimed at assessing the influence of the mode of delivery in term newborns on the first 24 hours metabolism of neonates.

Material and methods: This study was carried out on urine samples from 42 patients admitted to the Neonatal Intensive Unit and Neonatal Pathology of "S. Giovanni Calibita" Hospital Fatebenefratelli (Rome, Italy). According to the type of delivery, term neonates with similar gestational age and birthweight were divided in two groups: (1) born by spontaneous VD, (2) born by elective CS. Urine samples, collected at birth by a non-invasive method, were subjected to proton Nuclear Magnetic Resonance spectroscopy.

Results: CS newborns showed lower fatty acid omega oxidation, as evidenced by lower urinary excretion of dicarboxylic acids. This metabolic signature supports current evidence that babies delivered by CS have lower body temperature and perturbed thermogenesis. CS associates also with hypoglycaemia and altered endocrine profile, which linked to changes in central energy metabolic pathways (Krebs and Cori Cycles). Lung function may be reduced in infants born by CS, primarily due to delayed clearance of lung liquid, and surfactant insufficiency, which might be reflected in different

urinary excretion of myo-inositol and choline – two intermediates in lung surfactant metabolism.

Conclusion: Non-invasive urine metabolic phenotyping of children born by different mode of delivery provides relevant readouts to assess metabolic requirements associated with major physiological functions during this critical period of metabolic adaptation.

Keywords

Cesarean section, metabolomics, newborns, neonatal physiology, spontaneous delivery.

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Introduction

In the last years, the uncritical attitude towards cesarean section (CS) has been associated with the fast emergence of modern multifactorial diseases such as early pediatric obesity, type 2 diabetes and allergies [1]. Over the last 30 years a growing number of studies have demonstrated that babies born at term by vaginal delivery (VD) have significantly different physiology at birth than those born by CS, particularly when there has been no exposure to labour [2]. Hyde et al. suggested that VD initiates important physiological trajectories with subsequent influence on adult health [2].

There are a number of factors that might plausibly contribute to this programming, one of which is the hormonal surge or "stress response" of VD [2]. In particular, the authors discussed how CS links to changes in terms of lung function, reduced thermogenic response, altered metabolism, feeding, immune phenotype as well as altered blood pressure.

Another concept, the "hygiene hypothesis", suggests that an overly clean environment, especially in early childhood, may contribute to the development of several childhood diseases [3]. In particular, Neu and Rushing discussed how, during VD, the contact with the maternal vaginal and intestinal flora is an important source for the start of the infant's colonization, which is absent under CS [3]. It has therefore being argued that, if the intestinal microbiota develops differently depending on the mode of delivery, the postnatal development of the immune system might also be different.

The role of early life nutrition, including macro/micronutrient profiles, are subject to much attention for the prevention against obesity and other metabolic disorders later in life [4, 5]. The increased prevalence worldwide in child metabolic disease risk is foreseen to be influenced by various genetics, epigenetics, pre- and postnatal environmental factors [6-9]. Therefore, comprehensive understanding of the rapid physiological changes and their incidence on metabolic and nutritional requirements is needed to provide novel insights into improving the management of metabolic disorders and disease risk early in life. In particular, the study of neonatal metabolism using novel non-invasive technologies is envisioned to help in defining metabolic patterns related to optimal growth and development of tissues and organs, and individual predisposition to disease risk in childhood and adulthood [10].

Systems biology approaches, including metabolomics, are well suited for neonatal metabolic research due to the simple, safe and non-invasive approach that can be deployed on urine and stool samples [11-17]. Such data will serve as a reference to provide understanding of metabolic processes involved in developmental physiology, metabolic programming and nutritional requirements. This lies on building knowledge not only on the metabolic dysregulations, but also on the impact of different pre- and postnatal factors on the neonatal metabolism. Neonates undergo major physiological changes during the first days of life. Throughout the fetal life, homeostasis is achieved through the constant homeostatic placenta fluxes, which provide oxygen, glucose, nutrients, and fluid, and ensure removal of carbon dioxide and catabolic products [18]. The extrauterine survival implies the activation and the development of many physiological processes required for organ growth and establishment of functional biological

structures. For instance, neonatal physiology is primarily marked by important changes in the respiratory, cardiovascular, and immune systems, as well as hepatic and kidney functions. Postnatal physiological adaptations are also occurring very early at the level of the neural mechanisms, the gastrointestinal tract development and colonization by symbiotic microbiota [19], and body adjustment to thermic conditions due to the transition from intrauterine to extrauterine environment [20].

In relation to these short-term physiological changes in response to CS or VD, in the present study we aimed at assessing the influence of the mode of delivery, in healthy term human neonates, during their first day of life. The objective of the study was achieved through the study of urinary metabolic profiles measured by proton Nuclear Magnetic Resonance (^{1}H-NMR) spectroscopy. We here report and discuss our findings concerning central energy, fatty acids oxidation and host-bacterial metabolism in relation to the mode of delivery.

Materials and methods

Study population

This clinical trial was conducted in accordance with the ethical principles of Good Clinical Practice and the Declaration of Helsinki. This study was carried out on 42 patients admitted to the Neonatal Intensive Unit and Neonatal Pathology of "S. Giovanni Calibita" Hospital Fatebenefratelli (Rome, Italy). The study protocol (reference 58/2010) was approved by the local ethical committee on December 9th 2010. Written informed consent was obtained from the parents before enrolment in the study. According to the mode of delivery, term neonates were classified in two groups, namely born by spontaneous VD (n = 21) or born by elective CS (n = 21). Patients were matched for gender, gestational age and birthweight. Elective CS was performed either on request from the mother or due to a previous CS. In our study, to control for potential biases, mothers with infectious condition during pregnancy were excluded. None of the mothers had clinical signs of infection, nor altered laboratory values.

Experimental design and sample collection

The clinical data of each patient was recorded in the hospital registers under standard clinical practice. For each infant, urine spot samples were collected within 8 h of birth. With the aim to capture metabolic signatures that reflect immediate impact of the mode of delivery on the metabolism of the baby and to limit the influence of environmental factors (baby handing, feeding), we collected the urine samples within 8 hours of birth (**Fig. 1**). The urine was collected non-invasively using a sterile cotton ball placed in the disposable diaper. In absence of fecal contamination, about 1 mL of urine was aspired with a syringe and transferred to a sterile 2-mL vial. After collection, all the vials were immediately frozen and stored at -80°C until metabolomic analysis.

Sample preparation and ^{1}H-NMR spectroscopic analysis

Urines samples (500 µL) were adjusted to pH 6.8 using 100 µL of phosphate buffer solution (KH_2PO_4,

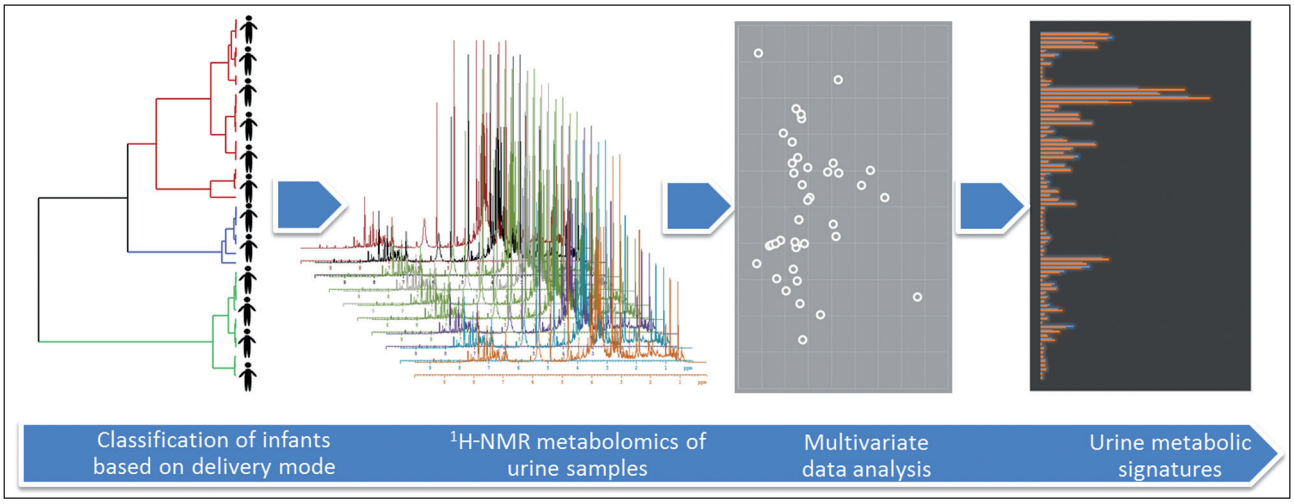

Figure 1. Experimental design of the clinical and metabolomics analysis.

final concentration of 0.2 M) containing 1 mM of sodium 3-(trimethylsilyl)-[2,2,3,3-2H$_4$]-1-propionate (TSP), and introduced into 5-mm NMR tubes. Urine samples were randomly measured on a Bruker AVANCE™ III 600 MHz spectrometer equipped with an inverse cryogenic probe. Urine samples were measured using a standard pulse sequence with water suppression, and 128 transients were collected into 96 K data points. ^{1}H-NMR spectra were processed using the software package TopSpin (version 2.1, Bruker, Germany). The FIDs were multiplied by an exponential weighting function corresponding to a line broadening of 0.3 Hz for urine spectra. The acquired ^{1}H-NMR spectra were automatically phased and baseline corrected, and referenced to the chemical shift of TSP at 0.00.

Chemometrics

Full resolution spectra incorporating data points within the 0.5-10.00 ppm region were imported and processed using in-house developed MATLAB® (The MathWorks® Inc., Natick, MA, USA) routines on unit-variance scaled NMR variables (i.e. each variable divided by its standard deviation). Following data normalization to total area under the curve, to correct for urine dilution, data were exported to SIMCA-P+ (version 14.0, Umetrics AB, Umeå, Sweden) for chemometric analysis. Initial data analyses were conducted using Principal Component Analysis (PCA) [21] in order to assess metabolic similarities between samples. Data were visualized by means of principal component scores, where each point represents an individual metabolic profile. NMR variables, e.g. metabolic concentrations, responsible for the differences between samples in the scores plot can be extracted from the corresponding loadings plot, where each coordinate represents a single NMR signal. In addition, a modification of Partial Least Squares Regression (PLSR) that removes all information orthogonal to the response variable during the fitting process was employed. This variant, Orthogonal Projection to Latent Structures (O-PLS) [22, 23] was applied to maximize the discrimination between sample groups focusing on differences according to variations during the first day of life. O-PLS Discriminant Analysis (O-PLS-DA) provides a way to filter out metabolic information (NMR spectral data) that is not correlated to the pre-defined classes. To test the validity of the model against over-fitting, the goodness of fit (R_X^2) and predictability (Q^2_Y) values of O-PLS-DA models were computed. To test the validity of the model against over-fitting, the cross-validation parameter Q^2Y was computed and the standard 7-fold cross validation method was used. Additional validation of the statistical modeling was performed using permutation testing. The mean of the distributions of the Q^2 parameters obtained using 500 random permutations is significantly different and lower than the experimental Q^2 parameters at 95% confidence interval using a one-tail t-test (**Fig. 2**). To highlight the weight of individual variables in the model, Variable Importance in Projection (VIP) was used, with a value above 1.5 used as a threshold by convention. In addition, a Pearson correlation coefficient was calculated with a p-value significant at 95% confidence interval (e.g. with $n = 42$, $r \geq 0.305$, $p < 0.05$).

Results

According to the type of delivery, term neonates showed similar gestational age and birthweight and the same gender distribution, as reported in **Tab. 1**.

For metabolomics urinary analysis, three metabolic profiles were discarded due to extreme dilution (one from spontaneous VD group, and two from CS group).

Urine spectra of neonates are characterized by the occurrence of a multitude of sharp resonances

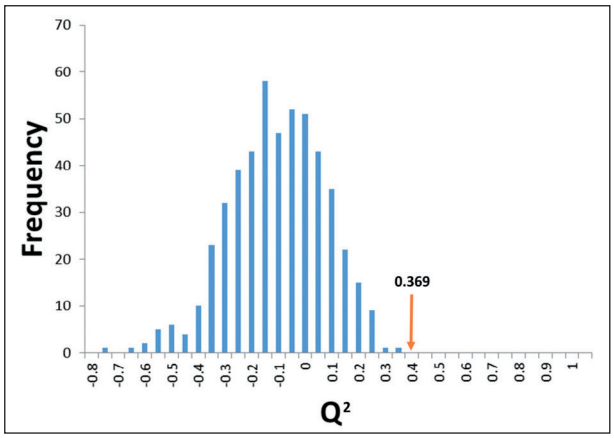

Figure 2. Histogram showing the Q^2 parameters obtained from O-PLS-DA models derived from ^{1}H-NMR spectra of urine using permutation testing (N = 500 permutations). The mean of the distributions is significantly different and lower than the experimental Q^2 parameter at 95% confidence interval using a one-tail t-test.

Table 1. Population characteristics.

Model type	N (males)	Birthweight (grams)	Gestational age (weeks)
VD	21 (11)	3,139.5 ± 440.4	38.9 ± 1.3
CS	21 (11)	3,259.2 ± 390.2	38.4 ± 1.1

VD: vaginal delivery; CS: cesarean section.

from organic acids including several aromatic compounds, amino acids, sugars and polyols, methylamines, purine and pyrimidine intermediates, alkaloids, creatine and creatinine, and osmolytes as previously reported [12].

Multivariate data analysis was performed on the metabolic profiles using PCA (**Tab. 2**). This multivariate data analysis explores the variance on the metabolomics that may explain differences between groups of samples. Initial inspection of the urine profiles in relation to the mode of delivery, gender or birthweight, did not reveal any major effect influencing the metabolic profile.

Additional supervised multivariate data analysis was conducted to explore the variance on the metabolomics data that may explain statistical differences between the groups of samples defined according to the mode of delivery (**Fig. 3**). Here, significant metabolite concentration differences were observed in the urine composition between babies born by spontaneous VD or CS, as noted by the O-PLS-DA model generated with one predictive and two orthogonal components (**Tab. 2**, where R^2X corresponds to the explained variance in the metabolomics data [urine metabolites], R^2Y to the explained group variance [delivery groups] and Q^2Y to the robustness of the model).

Inspection of the model coefficients enables the identification of urinary metabolites that showed differentiating patterns according to the mode of delivery (**Tab. 3**). VD newborns showed markedly

Table 2. Overview of model parameters.

Model type	Model parameters	R^2X, R^2Y, Q^2Y
PCA	4 principal components	0.4, NA, 0.04
PLS-DA	3 predictive components	0.23, 0.95, 0.52
O-PLS-DA	1 predictive and 2 orthogonal components	0.23, 0.95, 0.37

PCA: principal component analysis; PLS-DA: partial least squares discriminant analysis; O-PLS-DA: orthogonal projection to latent structures discriminant analysis; R^2X: explained variance in the metabolomics data (urine metabolites); R^2Y: explained group variance (delivery groups); Q^2Y: robustness of the model.
Partial Least Squares Regression (PLSR) that removes all information orthogonal to the response variable during the fitting process was employed.

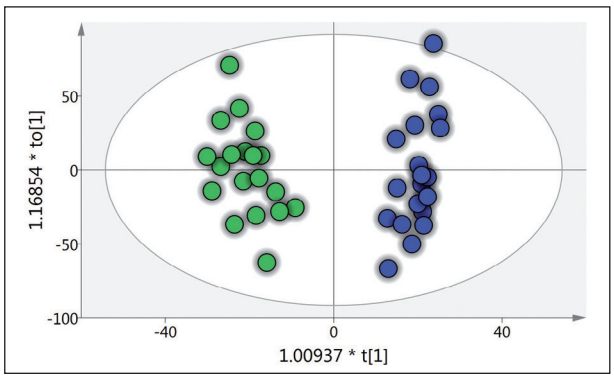

Figure 3. O-PLS-DA scores plot derived from ^{1}H-NMR spectra of urine. The cross-validated scores plot showed statistically significant separations between the urinary profiles obtained from CS (green) and VD (blue).
CS: cesarean section; VD: vaginal delivery.

Table 3. Overview of influential urinary metabolites discriminant between the two modes of delivery in term infants.

Metabolites	^{1}H-NMR metabolite signal (ppm)	Pearson correlation coefficient (r)	Mode of delivery with higher urinary concentration	Variable Importance in Projection (VIP)	Related biochemical processes
Choline	3.21	-0.41	CS	1.53	Cell membrane synthesis One carbon metabolism
Dimethylamine	2.71	0.40	VD	1.60	Choline metabolism
Formate	8.466	-0.41	CS	1.57	One carbon metabolism
Hippurate	7.85	-0.39	CS	1.57	Metabolism of phenolics
Histidine	7.02	-0.40	CS	1.54	Histidine metabolism
Myo-inositol	4.087	0.39	VD	1.56	Osmolyte Surfactant metabolism
Lactate	4.15	0.43	VD	1.77	Energy metabolism
Lysine	3.02	-0.50	CS	1.86	Lysine metabolism
Alpha-aminoadipate	1.63	0.42	VD	1.58	
N-acetyl-glycoprotein	2.06	0.48	VD	1.94	Glycoprotein
O-acetyl-glycoprotein	2.08	0.53	VD	2.06	
Oxaloacetate	2.37	0.38	VD	1.53	Urea cycle Krebs cycle
Beta-ketoadipate	2.429	0.28	VD	1.53	Fatty acid metabolism
Sebacate	1.26	0.40	VD	1.51	
Suberate	1.24	0.59	VD	2.18	

CS: cesarean section; VD: vaginal delivery.

higher urinary levels of two major dicarboxylic acids, suberate and sebacate, an intermediate in Krebs cycle, oxaloacetate, as well as lactate. This metabolic signature is pinpointing towards a different fatty acid oxidation metabolism. These urinary patterns in VD children were concomitant to lower urinary levels of histidine and lysine, but higher level of alpha-aminoadipate, a lysine precursor, when compared to CS infants. In addition, spontaneously delivered infants showed lower urinary content in the osmolyte choline, but higher urinary excretion in another osmolyte, inositol. Infants born vaginally showed a higher urinary excretion of N-acetyl-glycoproteins than infants born with CS. Furthermore, differential urinary excretion in bacterial related metabolites may reflect different host-microbe interactions. These metabolites included higher urinary excretion of beta-ketoadipate, dimethylamine, and lower levels of formate and hippurate in spontaneously born infants.

Discussion

Major physiological adaptations take place in the early postnatal period to cope with stress and cold exposure, respiratory adaptation, and switch of metabolic fuel selection from glucose to fatty acids. Metabolic changes captured by metabolomics were discussed in relation to CS differences for thermogenesis and perturbed energy metabolism at birth.

Influence of body temperature on thyrotropic hormone release and lipolysis in the newborn infant is well documented [24]. Marchini et al. reported how the change in environmental temperature as a result of extrauterine adaptation causes thermal stimulation of the infant's body surface. Metabolic adaption to this change encompasses the activation of hypothalamic-pituitary thyrotropin axis, and increased hormone release that induces a stimulation of lipolysis, and increased blood plasma free glycerol [24]. There is compelling evidence that babies delivered by CS, compared to VD babies, have a significantly lower body temperature after birth, which may link to perturbed thermogenic response [25]. Such physiological differences are supported by pre-clinical observations describing how CS reduces the capacity for non-shivering thermogenesis due to reduced levels of uncoupling protein-1 (UCP1) in the brown adipose tissue (BAT), and altered plasma thyroid hormone concentrations [26, 27]. BAT can dramatically increase metabolic rate and dissipate large amounts of stored lipids in a relatively short time once activated, which is achieved through fatty acid oxidation [28]. The oxidation of lipids can be achieved through beta and omega oxidation. In the present study, the higher urinary excretion of major dicarboxylic acids in VD newborns (i.e. sebacic, suberic and keto-adipic acids) suggests higher activation of omega oxidation, and therefore higher thermogenesis rate. In particular, dicarboxylic acids are end products of non-beta oxidation of medium chain fatty acids that occur outside the mitochondria in smooth endoplasmic reticulum of primarily liver and kidney cells. As newborns do not have the ability to shiver to cope with postnatal cold stress, those dicarboxylic acid changes could be assigned to activation of omega oxidation for thermic energy production. Omega oxidation of fatty acids is generally a minor catabolic pathway, but it becomes more important when beta oxidation is limited for instance when there is not enough carnitine available [12]. In newborns, fat may be underused as an energy substrate due to the inhibition of mitochondrial beta-oxidation, since fatty acid beta-oxidation involves carnitine-dependent enzyme activities.

Since glucose oxidation alone cannot fulfill the energy needs of the neonate in the immediate postnatal period, lipolysis is markedly activated after birth to mobilize free fatty acids and glycerol for oxidation and gluconeogenesis, respectively. Other clinical investigations have extensively described how postnatal metabolic adaptations and central energy metabolism are influenced by the mode of delivery. For instance, CS is associated with hypoglycaemia [29, 30] or altered endocrine metabolism, which subsequently influence energy metabolism [31]. As a consequence in changes in lipolysis and fat oxidation, concomitant changes in central energy metabolic pathways were pinpointed by the observation of higher urine levels in oxaloacetate and lactate in VD babies. Oxaloacetate is an intermediate of the Krebs cycle that may reflect higher fatty acid beta oxidation in our study. Lactate is the main end product of anaerobic glucose metabolism, and more likely reflects a change in the Cori cycle in the muscle. It is known that Cori cycle shuttles lactate to the liver where the nitrogen enters the urea cycle for gluconeogenesis. Bird et al. reported how umbilical plasma concentrations of thyroxine and triiodothyronine were significantly higher, whilst cortisol and thyroid stimulating hormone (TSH) concentrations were lower after CS compared to VD [31]. The authors discussed

how the labour reduces plasma thyroid hormone concentrations at birth in association with a rise in cortisol, which may subsequently lead to major changes in CS child metabolism during the first days of life, including reduced lipolysis in the neonate [24, 32]. Our current findings on lipid metabolism and energy fuels in CS are in agreement with these physiological observations.

CS was associated with lower urinary loss in glycoproteins as well. It is well established that in human urine Tamm-Horsfall proteins are the most abundant proteins excreted, and therefore this pattern suggests that kidney functions are influenced differently according to the mode of delivery, and may contribute to the different urinary patterns of other metabolites, including amino acids or hippurate, as discussed hereafter. For instance, additional changes in the urinary excretion of two essential amino acids, lysine and histidine, may suggest perturbation of protein metabolism, specific enzymatic activities in lysine and histidine metabolism or kidney functions, as discussed previously in preterm infants [33]. For instance, Hao et al. previously reported lower urinary levels in preterm infants for both these amino acids, discussing their importance for calcium, lean mass and hormone metabolism [33, 34]. Here, we observed higher urinary loss related to CS in term infants, which may be potentially linked to different postnatal metabolic responses, including different kidney functions, or metabolic enzymatic activities as suggested by the different concentration in alpha-aminoadipate, an intermediate in lysine metabolism. Furthermore, hippurate metabolism is closely intertwined with kidney functions, where glycine is conjugated to benzoic acid. This metabolite has been previously documented as a relevant marker of renal clearance [35], and may further support changes in glomerular filtration according to the mode of delivery. It can be argued that hippurate urinary changes may be a result of the child exposure to mother or environmental microbiome, which are of major importance in the field of study on CS [36].

The occurrence of relatively high urinary concentration of the osmolytes myo-inositol and choline in newborn urine needs to be paralleled with the postnatal maturation of organ and tissue function. On the one hand, myo-inositol is a key component incorporated into lung cell membranes and serves as a precursor for surfactant synthesis. It has been reported to be deficient in premature infants [37]. Moreover, lung function is significantly compromised in infants born by CS compared to VD, primarily due to delayed clearance of lung liquid, pulmonary hypertension and surfactant insufficiency [36]. Therefore, the lower urinary level of myo-inositol in CS children may reflect differences in metabolic requirements (e.g. surfactants) or biosynthesis/catabolism primarily by the kidney [38]. On the other hand, choline is also critical for *de novo* lung lecithin synthesis [39]. Lung underdevelopment, lung hypoplasia, abnormal lung water metabolism, inflammation, and pulmonary surfactant deficiency or dysfunction play a variable role in neonatal lung diseases [39]. Therefore, our observations may pinpoint towards a different phospholipid metabolism with CS. Formate, higher in CS newborn urine, is with choline a key intermediate in the methyl transfer metabolism. Methyltransferase pathway is a key component of cellular metabolism, involved in synthesis of purines, pyrimidines, and methylation of a number of substrates, proteins, DNA, RNA and indirectly expression of a number of genes [40]. Dimethylamine is also high in CS infant urine, and may have both endogenous (e.g. transmethylation of methylamine derived from sarcosine or glycine) and exogenous origin (e.g. microbial and dietary sources). These concomitant changes may indicate a more profound perturbation of choline metabolism, including higher methyltransferase pathway activities related to the maturation of hepatic functionalities and/or brain metabolic requirements.

Conclusion

With increasing number of CSs each year, this is an area that requires more research attempting to understand the mechanisms by which metabolic programming may impact life-long health outcomes. Here we report that urinary metabolic fingerprints of infants born by CS and VD reflect important short-term adaptations of the infant metabolism. Urine analysis is well suited to provide a time-averaged representation of recent homeostatic controls, whilst being a biofluid accessible non-invasively. Differences for thermogenesis and perturbed energy metabolism at birth in CS infants have a profound influence on central biochemical pathways involved in fatty acid oxidation, gluconeogenesis, surfactant biosynthesis, and kidney functions. Our study has limitations, since this is a small, pilot study with concomitant limitations of sample size and risk of false positives, so metabolic signatures should be further studied in future clinical research. Non-

invasive urine metabolic monitoring is foreseen to provide relevant readouts to assess metabolic requirements and to guide nutritional management of infants' growth and development.

Declaration of interest

FPM and SR are current employees of Nestlé, a food and beverage company. At the time the work was performed, LDS and SCo were employees of Nestlé as well. The other Authors declare that there is no conflict of interest.

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