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Urinary ^{1}H-NMR and GC-MS metabolomics predicts early and late onset neonatal sepsis

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ABSTRACT

The purpose of this article is to study one of the most significant causes of neonatal morbidity and mortality: neonatal sepsis. This pathology is due to a bacterial or fungal infection acquired during the perinatal period. Neonatal sepsis has been categorized into two groups: early onset if it occurs within 3–6 days and late onset after 4–7 days. Due to the not-specific clinical signs, along with the inaccuracy of available biomarkers, the diagnosis is still a major challenge. In this regard, the use of a combined approach based on both nuclear magnetic resonance (^{1}H-NMR) and gas-chromatography-mass spectrometry (GC-MS) techniques, coupled with a multivariate statistical analysis, may help to uncover features of the disease that are still hidden. The objective of our study was to evaluate the capability of the metabolomics approach to identify a potential metabolic profile related to the neonatal septic condition. The study population included 25 neonates (15 males and 10 females): 9 (6 males and 3 females) patients had a diagnosis of sepsis and 16 were healthy controls (9 males and 7 females). This study showed a unique metabolic profile of the patients affected by sepsis compared to non-affected ones with a statistically significant difference between the two groups (p = 0.05).

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1. Introduction

Sepsis is a dysfunction affecting different organs caused by infectious microorganisms and induced by mediators of inflammation that cause alteration of the immune, inflammatory and coagulative equilibrium. Sepsis is divided into early onset sepsis (EOS), which takes place in the first 72 hours (according to some authors within the first week of life) and late onset sepsis (LOS), which begins after 72 hours and is usually caused by nosocomial pathogens [1]. In the United States, the incidence of early onset sepsis, assessed in two different studies (Centers for Disease Control and Prevention and National Institutes for Child Health and Development) was in both studies approximately 3300 cases with 400 deaths per year [2]. Moreover, the rates of infection and mortality increase with the decrease in gestational age and birth weight. The most common etiologies of EOS are *E. coli* and group B streptococcus (GBS) (~23% and 47% of cases, respectively). For LOS, coagulase-negative staphylococci have emerged as major pathogens causing 39% of cases. *E. coli*, *S. aureus*, *Enterococcus* spp. and GBS are important pathogens during the neonatal period, although there is wide regional variability. However, during the neonatal period, *Klebsiella* spp., *Enterobacter* spp., *P. aeruginosa* and *S. marcescens* emerge as etiologies of LOS which are rare pathogens within seven days of life [3,4]. Worldwide, neonatal sepses are the cause of approximately 36% of the four million deaths of neonates every year. Neonatal sepsis onsets in a subtle way and clinical signs at the beginning may be vague and aspecific (not looking well, not eating well, not breathing well). Early diagnosis is a challenge to pediatricians and for this reason in the last few decades several studies have been conducted in an attempt to find a test that satisfies the criteria to make it the ideal marker for EOS in the neonate. In this field, metabolomics appears to be quite promising [5]. Recent studies show the efficacy of this method in providing significant information leading to an early diagnosis of numerous neonatal pathologies [6]. Metabolomics is a holistic approach that studies the complete

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set of low-molecular-weight metabolites contained in human body fluids [6]. The analysis of the metabolic profile observable in a body fluid allows the immediate identification of changes in the composition of endogenous and exogenous metabolites that may be correlated with specific pathophysiological states, gene expression and interaction with the environment [7]. To date, very few publications have dealt with metabolomics and sepsis in adults and children [8] and none in neonates. In our work we performed a metabolomic analysis to assess variations of metabolites preceding the onset of early and late sepsis in neonates for the purpose of identifying a metabolic state leading to the onset of infection.

2. Materials and methods

2.1. Patients

This study was carried out on urine samples from two groups of patients admitted to the four centers participating: the Neonatal Intensive Care Unit (NICU) of the University of Cagliari, the NICU of the San Matteo General Hospital (Pavia), the NICU of the University of Palermo and Department of Maternal, Fetal and Neonatal Health in the C. Arrigo Children's Hospital (Alessandria). The single ethical committees approved the study protocol and written informed consent was obtained from the parents before enrolment in the study. The first group consisted of 9 newborns (6 males and 3 females with mean gestational age 29.1 weeks) who had received a diagnosis of sepsis and the second group consisted of 16 healthy controls (9 males and 7 females with mean gestational age 34.6 weeks). Among the septic patients, there were 5 cases of EOS (4 males and 1 female), with a mean age of 2.4 days at the time of infection and 4 cases of LOS (2 males and 2 females) with a mean age of 12.2 days at the time of infection. All newborns analyzed in this experimental protocol have survived. The clinical data of each patient were recorded in the hospital registers. Urine samples (2–3 mL) were collected at birth and at regular intervals during the first month of life, then aspired with a syringe and transferred to a sterile 15 ml Falcon tube. Due to a low volume of urine, NMR evaluations were performed only in 14 healthy controls and 7 septic newborns (2 EOS, 5 LOS). The tubes were then split in two vials containing 10 μL of a solution of sodium azide (NaN_3) to prevent bacterial contamination and then stored at −80°C awaiting the metabolomic analysis.

2.2. Metabolomic analysis

2.2.1. Reagents

All chemicals used in this study were of analytical grade. Dodecane was used as internal standard and pyridine and hexane were used as a solvent (Sigma, St. Louis, MO, USA). Methoxyaminehydrochloride, N,O-bis(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane (BSTFA + TMCS) and urease were purchased from Sigma (St. Louis, MO, USA). Deuterium oxide (D_2O, 99.9%) was purchased from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). Sodium 3-trimethylsilyl-propionate-2,2,3,3,-d_4 (TSP, 98 atom% D) was acquired from Sigma-Aldrich (Milan, Italy).

2.2.2. GC-MS urine sample preparation

After thawing the urine samples on ice, they were centrifuged for 10 minutes at 14000 rpm then 150 μL of supernatant were transferred into an Eppendorf tube containing 1 mg of urease. Samples were sonicated for 30 minutes at 37°C and then centrifuged for 10 minutes at 13200 rpm. The supernatant was separated and dried under nitrogen overnight and was derivatizated with 50 μL of a solution of methoxyamine in pyridine (10 mg/mL). After 17 h 100 μL of BSTFA + TMCS was added and after 1 h samples were resuspended with 600 μL of hexane containing dodecane at 5 mg/L.

2.2.3. GC-MS analysis

One microliter of derivatized sample was injected splitless into a 6850 gas chromatograph coupled with a 5973 Network mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) equipped with a 30 m ×0.25 mm ID, fused silica capillary column, which was chemically bonded with 0.25 μm DB5-MS stationary phase (J&W scientific, Folsom, CA, USA). The injector temperature was 200°C. The gas flow rate through the column was 1 mL/min. The column initial temperature was kept at 50°C for 10 min. Then the temperature was increased from 50 to 300 at 10°C/min and held at 300°C for 10 minutes. The transfer line and the ion source temperatures were respectively 280°C and 180°C. Ions were generated at 70 eV with electron ionization and were recorded at 1.6 scan/sec over the mass range *m/z* 50–550. GC-MS data analysis was conducted by integrating each resolved chromatogram peak and normalizing the area for the corrected total area of the chromatogram. These peaks were examined for their mass spectra and identification of the peaks was attempted using the NIST08 library after deconvolution with AMDIS.

2.2.4. NMR urine sample preparation

Samples of 1 mL of thawed urine were centrifuged at 12,000 rpm/min for 10 minutes at 4°C. An aliquot of 630 μl was then withdrawn from the supernatant and 70 μl of a 1.5 M phosphate buffer solution pH 7.4 with a final concentration of 1 mM TSP (trimethylsilyl propanoic acid) was added. Each sample was stirred for 60 seconds and transferred into a 5 mm NMR tube for analysis.

2.2.5. NMR analysis

The spectra were acquired at 499,839 MHz using a Varian Unity Inova 500 MHz spectrometer. The experiments were carried out using a standard temperature of 27°C, a 1D NOESY sequence with a 90° pulse of 10.4 ms, an acquisition time of 2 s, 128 transients with a spectral width of 6000 Hz. Once the spectra were acquired, phase and baseline corrections were performed (Version 7.1.2, Mestrelab Research S.L.). Afterwards, the ^{1}H-NMR spectra were reduced to consecutive integrated spectral regions (bins) of equal width (0.04 ppm) corresponding to the region 0.5–9.5 ppm. The spectral region between 4.70–5.14 and 5.50–6.00 were excluded from the analysis to remove the effect of variations in the presaturation of the residual water and urea resonances. Bucketing was performed by MestReNova. The integrated area within each bin was normalized to a constant sum of 100 for each spectrum to minimize the effects of variable concentration among different samples. The final data set was imported into the SIMCA-P+ program (Version 13.0, Umetrics, Umeå, Sweden).

2.2.6. Statistical approaches

An orthogonal partial least square discriminant analysis (OPLS-DA) of GC-MS and NMR data was performed using the SIMCA software package (version 13.0, Umetrics, Umea, Sweden). OPLS-DA is a data visualization method that is useful for observing grouping within multivariate data. This supervised analysis uses linear regression, where the class memberships of samples are included in the calculation. OPLS is able to rotate the projection so that the model focuses on the effect of interest allowing a better distinction between groups. The validity of the OPLS-DA model is assessed by statistical parameters: the correlation coefficient R^2 and the cross-validation correlation coefficient Q^2. R^2 represent the accuracy of the fit to the model and Q^2 reveals the grade of predictability of the model. From a supervised analysis it is possible to obtain the set of VIPs, "important variables on the projection", the metabolic variables that contribute to the characterization of groups.

3. Results

GC-MS is considered the gold standard in metabolite detection and quantification; in fact, the sensitivity of MS is in the pico/nano molar range, whilst proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy is also considered a major tool for analyzing a large number of molecules simultaneously with an analytical sensitivity ranging 1–10 μmol/L. However, below this cutoff, the detection and quantification of metabolites is still unreliable. The use of these techniques allows the identification of different types of metabolites depending on their concentration and chemical structure; therefore, it may be appropriate to use both methods to cover the largest number of molecules. The GC-MS coupled with ^{1}H-NMR analysis showed important differences among neonates with a septic condition compared to the controls. The metabolites responsible for those differences were identified from the urine samples collected at birth from the EOS group and within 72 hours prior the onset of infection for the LOS group. The resulting fingerprint may be considered a predictive tool of the septic condition.

3.1. GC-MS

A comparison between the GC-MS chromatograms of urine samples collected from control and septic newborns is shown in Fig. 1 and the most important metabolites are reported in Table 1.

As it can be seen, there are significant qualitative and quantitative differences between the two samples, the neonatal sepsis

Table 1
Urinary most relevant upregulated or downregulated metabolites in the septic group, compared with the control group.

Compound	t_R (min)	Trend
Lactate	15.58	↑
Glucose	27.21	↑
Maltose	34.58	↑
Ribitol	25.29	↓
Ribonic acid	25.92	↓
Pseudo uridine	31.16	↓
2,3,4-trihydroxybutyric acid	23.20	↓
2-ketogluconic acid	25.81	↓
3,4-dihydroxybutanoic acid	21.70	↓
3,4,5-trihydroxypentanoic acid	24.51	↓

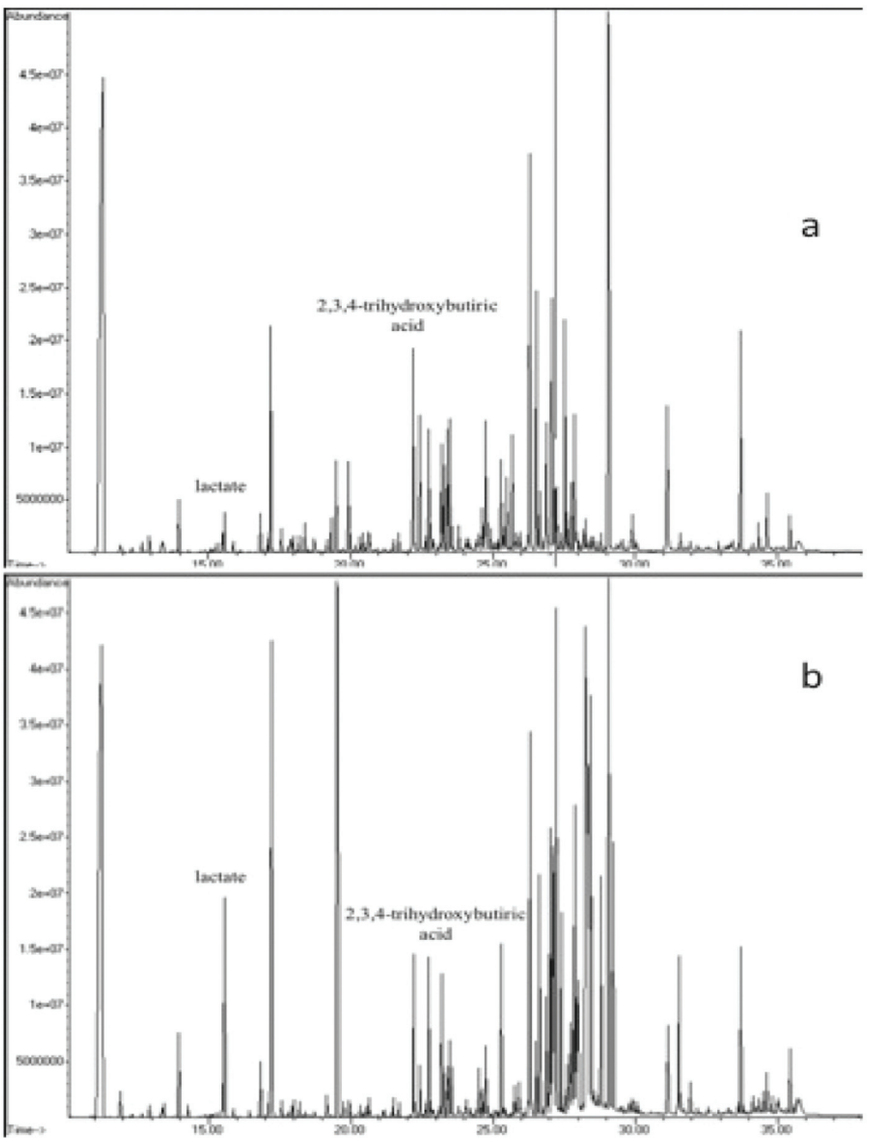

Fig. 1. GC/MS chromatograms of urine samples from (a) control and (b) septic subjects.

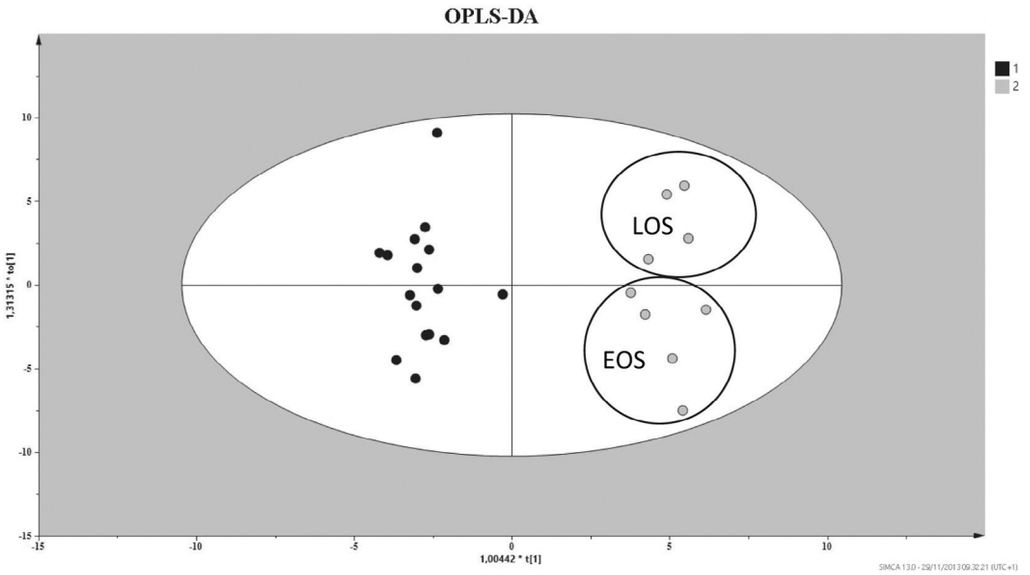

Fig. 2. OPLS-DA scores plot of control (filled circles) vs septic (shaded circles) urine samples.

chromatogram being characterized by peaks higher in number and intensity with respect to those of controls.

The OPLS-DA analysis evidenced a clear separation between the groups (Fig. 2). In fact, controls were homogenously located in the left square of the plot, while septic samples were distributed in the right square. To identify the variables (metabolites) separating the two groups, the spectra data of the corresponding urine samples were deconvoluted using AMDIS-NIST08, and the resulting data were analyzed using an OPLS-DA classification model. The OPLS-DA model was generated with predictive components (T) and orthogonal components (T_o) to discriminate between groups (sepsis vs control) reflecting a high goodness of fit and predictability as indicated by an R2Y value of 0.96 and a Q^2 value of 0.62, respectively with a CV-ANOVA p-value of 0.003.

The analysis of the corresponding loading plot revealed variables (i.e. metabolite retention times) of importance for the clustering, thus allowing the identification of metabolites responsible for the observed variance: metabolites located in the left part were positively correlated with control samples, whereas those located on the right were positively correlated with septic samples. The variables significantly contributing to the separation of the septic samples included several metabolites such as maltose glucose and lactose, whereas control samples were characterized by ribitol, ribonic acid, pseudouridine, 2,3,4-trihydroxybutyric acid (THBA), 2-ketogluconic acid, 3,4-dihydroxybutanoic acid and 3,4,5-trihydroxypentanoic acid.

3.2. NMR

A high-resolution ^{1}H-NMR spectra comparison between the urine samples collected from control and septic newborns is shown in Fig. 3, and the dominant metabolites identified belonging to the aliphatic and carbohydrate areas are also reported in Table 2. To be noted is that few samples were not analyzed due to the paucity of the sample. The assignment of major resonances was performed on the basis of data published in the literature and using the Chenomx database (Edmonton, Alberta, Canada).

In this case the OPLS-DA model was able to clearly differentiate between neonatal sepsis and controls, reflecting a high goodness of fit and predictability as indicated by an R2Y value of 0.85 and a Q^2 value of 0.43 respectively, with a CV-ANOVA p-value of 0.05 (Fig. 4).

The variables significantly contributing to the separation of the septic samples included several metabolites such as acetate, glycine, glucose, acetone, lactate and lysine, whereas control samples were characterized by citrate and creatinine. The analysis of the corresponding loading plot revealed variables (i.e. bins) of importance for the clustering, thus allowing the identification of metabolites responsible for the observed variance: metabolites located in the left part were positively correlated with control samples, whereas those located on the right were positively correlated with septic samples. The loadings corresponding to 3.4, 3.36, and 1.36 ppm showed positive values, indicating that septic samples contained relatively higher contents of glucose and lactate with respect to the control group (Fig. 2b). On the contrary, control samples appeared to be richer in citrate and creatinine (2.56, 4.08 ppm). Furthermore, other bins, belonging to unidentified metabolites, were found to be important for sample clustering (e.g., 1.16 belonging to the pathological group). It is also worth noting that a deeper

Table 2
^{1}H-NMR chemical shift of the urinary most relevant metabolites identified in the control and septic groups. The trend identifies the behavior of septic newborns, in comparison with controls.

Compound	Group	^{1}H (ppm) [a]	^{1}H multiplicity [b]	Trend
Acetate	CH_3	1.92	s	↑
Acetone	CH_3	2.24	s	↑
Citrate	CH_2	2.54	d	↓
	CH_2	2.67	d	
Creatinine	N-CH_3	3.05	s	↓
	N-CH_2	4.06	s	
Glycine	CH_2	3.57	s	↑
Lactate	CH_3	1.33	d	↑
	CH	4.1	q	
Lysine	αCH	3.74	t	
	βCH_2	1.89	m	
	γCH_2	1.45	m	↑
	δCH_2	1.69	m	
	εCH_2	3.01	t	
Glucose	C2H	3.23 3.52	dd	
	C3H,C6H	3.73	m	
	C5H	3.46	m	
	C4H	3.4	m	↑
	C5H,C6H	3.82	m	
	C6H	3.88	dd	
	C1H	5.24 4.64	d	

[a] ^{1}H chemical shifts are reported with respect to TSP signal (0.00 ppm).
[b] Multiplicity definitions: s, singlet; s, doublet; t, triplet; dd, doublet of doublets; m, multiplet.

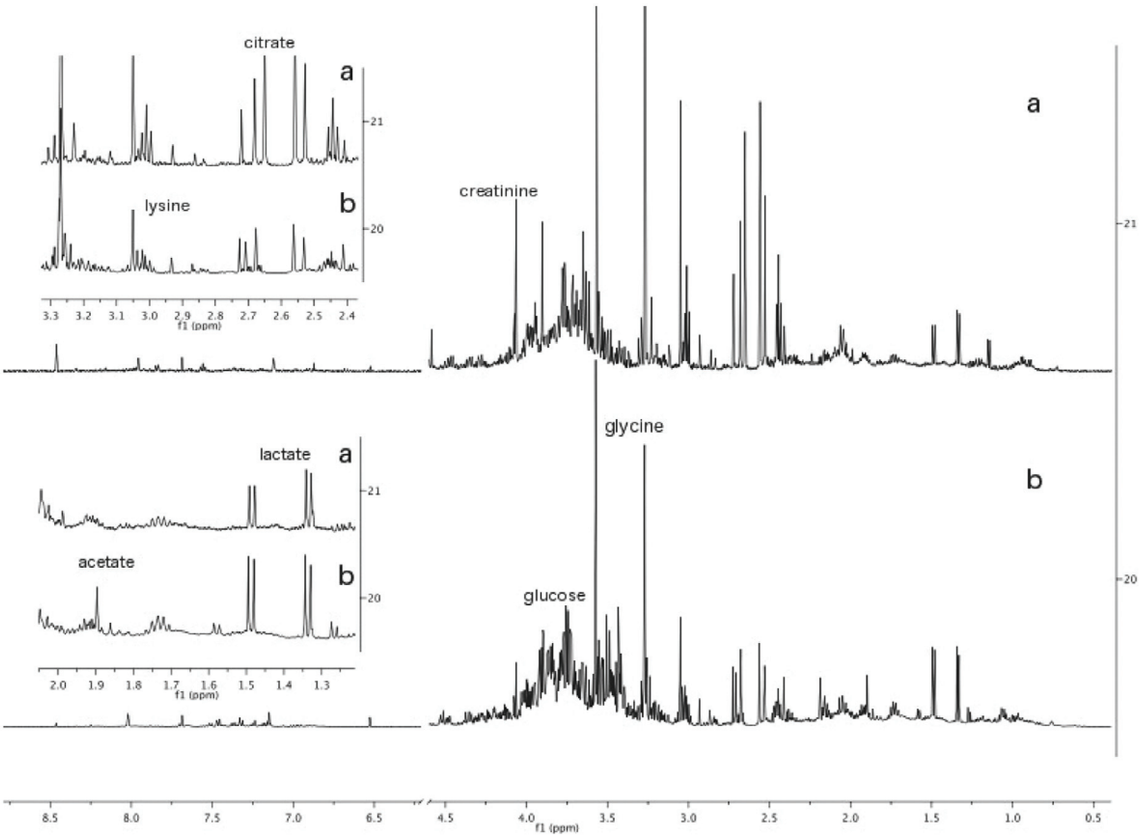

Fig. 3. ^{1}H-NMR spectra of urine samples from (a) control and (b) septic subjects with main assignments.

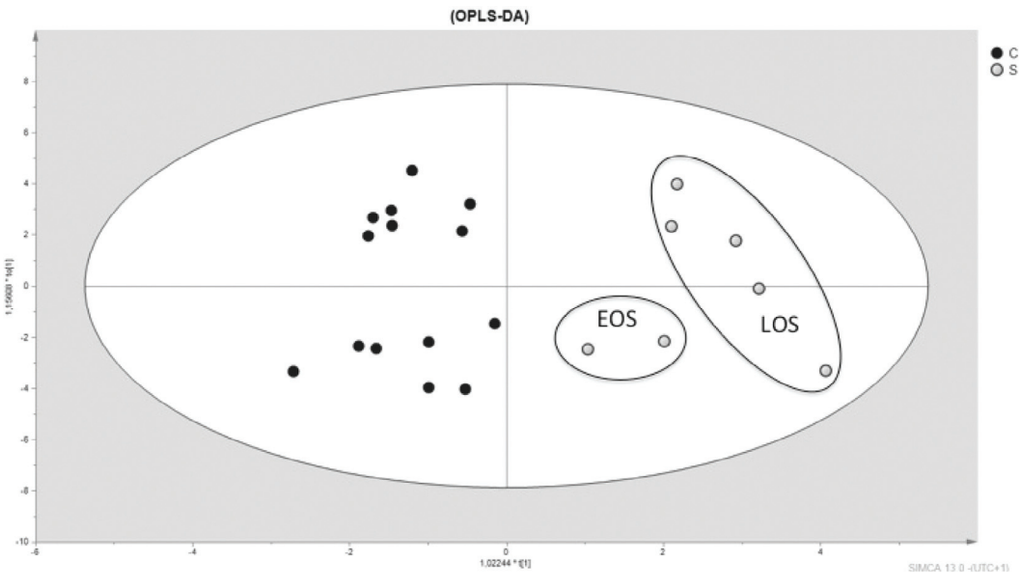

Fig. 4. OPLS-DA scores plot of control (filled circles) vs septic (shaded circles) urine samples.

examination of the score plot showed that EOS samples were clustered with respect to the LOS samples. The relation between the metabolic profile of the EOS and the metabolic profile of the LOS groups was not possible because of the low number of samples.

4. Discussion

Although biomolecular knowledge on the pathophysiology of neonatal sepsis has improved over the years, no markers making early diagnosis possible have yet been identified, nor have prognostic biomarkers of high sensitivity and specificity been found. Several studies have shown that for every hour of delay in administration of an appropriate antibiotic therapy there is a 7% increase in mortality [9]. The need for a highly sensitive and predictive test in the neonatal period for identifying and correctly classifying the maximum number of septic neonates makes metabolomics a method of great diagnostic potential. To our knowledge, this is the first work described in the literature that has analyzed with

metabolomics the urine metabolic profiles in septic neonates and controls for the purpose of defining the metabolic patterns associated with this pathology. From the very first analysis, performed both with mass spectroscopy and NMR on urine samples of the four participating centers, it was possible to identify by multivariate analysis a different metabolomics pattern between classes of neonates having a different clinical outcome (sepsis versus control). From this profile the septic neonates were significantly separated from controls. Moreover, the samples from EOS neonates were separated from those of LOS (although overall the samples were too few to arrive at a final assessment) (Figs. 2 and 4). In this study, metabolomics led to identifying the molecules responsible for the differences in the metabolic profiles, among which glucose, lactate and acetate, the urine content of which had increased in septic neonates compared to controls, while THBA, ribitol, ribonic acid and citrate had decreased. These results agree with recent works published in the literature. In particular, in our study the presence of acetone ketone bodies in the urine of the septic group suggests a compensatory reaction to a reduced level of ATP. It is interesting to see that also in works on metabolomics in the literature, conducted both on animals and humans, there were increases in metabolites (in plasma and urine) which are part of the oxidative metabolism of fatty acids, such as the ketone bodies (hydroxybutyrate, acetoacetate), in septic subjects [8,10–12]. Lin et al. suggest that the increase in acetoacetate and hydroxybutyrate in septic rats may be correlated with major oxidation of fatty acids. As the major source of energy, fatty acid oxidation is significantly enhanced to meet the energy requirement [13], and ketone bodies, which are important products of fatty acid oxidation, increase and accumulate accordingly [10]. Lactate and glucose were also found to have increased in the urine of septic patients compared to controls both in our study and other works [8,10,12,14] while citrate, a metabolite of the Krebs cycle, had decreased. These results can be explained by taking into account that sepsis corresponds to a systemic inflammation associated with mechanisms such as hypoxia and oxidative stress. Sepsis per se induces an impairment in energy production at the cell level owing to the blocking of the pyruvate dehydrogenase complex, the enzyme intimately involved in the production of lactates from which depend access of pyruvate into the Krebs cycle [15,16]. It is known that during septic shock there is an increase in glucose turnover through glycolysis with an increase in the levels of lactate of anaerobic production [17]. Briefly stated, the alterations in the glucose metabolism in acute critical conditions can be seen as a redistribution of glucose consumption from the mitochondrial oxidative phosphorylation to other metabolic pathways, such as the production of lactate and the pentose phosphate pathway. In our metabolomic study of the urine of septic neonates, this is confirmed by an increase in the levels of glucose and lactate and a decrease in the metabolites of the pentose phosphate pathway such as THBA, ribitol and ribonic acid. It is to be emphasized that the two methods take a "photograph" of the phenomenon in a way that is only partially superimposable, as ^{1}H-NMR spectroscopy does not require sample modification and allows the simultaneous identification of hundreds of metabolites with an analytical sensitivity in the range of 1–10 mmol/L. However, below this cut off, the detection and quantification of metabolites is still unreliable whilst GC/MS is also considered the gold standard in metabolite detection and quantification; depending on the metabolite, the sensitivity of MS is in the pico/nano molar range.

5. Conclusions

Present-day methods and procedures for the diagnosis of neonatal sepsis are hindered by low sensitivity and long response times. They are not capable of meeting the need to proceed to with rapid, timely and efficacious therapeutic treatment. In this study, thanks to metabolomics, it was possible to identify the metabolites responsible for the differences between septic neonates and controls: for EOS neonates at the time of birth and for the LOS neonates within 72 hours preceding the clinical onset. These biomarkers may be considered as early and reliable predictors of neonatal sepsis since the urine samples used were collected previous to the onset of infection. The data emerging from this study suggest that in the near future metabolomics will make possible to identify an early metabolic profile of sepsis and thus begin earlier, more targeted and effective treatments. However, in the next few years these results will have to be supported by further studies to increase the numbers of samples and thus arrive at a more complete and significant picture of the metabolic differences between septic neonates and controls.

Conflict of interest

The authors have no conflict of interest to declare.

Role of the funding source

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