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^{1}H NMR-based metabolomic analysis of urine from preterm and term neonates

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1. ABSTRACT

Metabolomics is a technique used to non-invasively determine metabolic status of an organism. Aim of our study was to analyze urinary metabolic profiles in term and preterm infants in order to identify gestational age-related metabolic differences and to predict metabolic maturity at birth. Twenty-six healthy term infants and 41 preterm infants were prospectively enrolled. A urine sample was collected non-invasively within the first hours of life. Samples were analyzed by proton nuclear magnetic resonance (^{1}H NMR) spectroscopy and NMR urine spectra were analyzed by multivariate statistical analysis. Distinct metabolic patterns were found between term infants and preterm infants, as well as between preterm infants of 23-32 weeks' gestation and those of 33-36 weeks' gestation. Individual metabolites discriminating between these groups were hippurate, tryptophan, phenylalanine, malate, tyrosine, hydroxybutyrate, N-acetyl-glutamate, and proline. Metabolomic analysis revealed distinct urinary metabolic profiles in neonates of different gestational ages, and identified the discriminating metabolites. This holistic approach appears to be a promising tool for investigating newborn metabolic maturation over time, and might lead to a tailored management of neonatal disorders.

2. INTRODUCTION

Preterm birth, defined as a birth occurring before the 37th weeks of gestation, is considered an abnormal outcome for a fetus. The frequency of preterm births is approximately 13% in the USA and 5-9% in Europe and other developed countries (1,2). Preterm infants are a heterogeneous category of neonates having relevant differences in organ system maturation, and degree of viability. In fact, babies born near term may have relatively few short- or long-term consequences, whereas survival of extremely preterm infants is threatened, as is the subsequent quality of life due to the difficulty in adaptation to extra-uterine life. Preterm births account for 75% of perinatal mortality and more than 50% of the long-term morbidity (3). As a result of prematurity-related complications, preterm infants may require medical treatment or even intensive care during the first weeks or months of life. Gestational age (GA) at birth is a commonly used parameter to evaluate the degree of development of preterm infants, and to optimize the clinical management of them. However, this parameter is only one of the factors affecting the neonate's metabolic maturation, which is also influenced by multiple prenatal and perinatal factors. A better knowledge of these issues is potentially of great

importance to improve and individualize the clinical management of prematurely born babies. The recently developed approaches of genomics, proteomics and transcriptomics refer to analysis technologies having a holistic view of genes and proteins. In numerous contexts, however, these tools have not been able to provide a complete set of biomarkers useful in diagnosis, therapy, and preventing drugs' side effects. This requirement may be further met by metabolomics, the youngest of the "-omics" sciences (4). Metabolomics is a functional genomics tool concerned with the high-throughput identification and quantification of metabolic compounds. The metabolomic approach is based on the analysis of multiparametric metabolic profiles typically generated by nuclear magnetic resonance (NMR) spectroscopy (5), or by mass spectrometry (6). Recent studies have demonstrated the ability of proton NMR (^{1}H NMR) to accurately profile the metabolic makeup of a biofluid from both a qualitative and quantitative point of view. Spectroscopic data are typically analyzed using chemometric and pattern recognition techniques to extract latent metabolic information and enable sample classification and biomarker identification. In recent years, increasing clinical application of NMR-based metabolomics has been observed, particularly in the diagnosis of adult pathological conditions (7-9). ^{1}H NMR analysis of human biofluids provides characteristic metabolite "fingerprints" that are affected by the basic mechanism, severity, and location of a pathological lesion (5,10-13). In particular, urine has a unique and characteristic biochemical composition that is a function of the combination of factors such as genotype, physiological state, disease state, nutritional state, environment, etc. This biofluid may provide information not only on the overall metabolic status of an individual, but also on the metabolic changes associated with renal or urinary disorders. Due to its characteristics and simple non-invasive methods of collection, urine is particularly suited for metabolomic analysis in humans, even in tiny babies. So far, to the best of our knowledge, few studies focusing on NMR analysis of urine have been carried out in premature babies (14-16), but no reports have been published on the metabolomic analysis of urine in preterm infants. In the present study, a ^{1}H NMR-based metabolomics approach was used to analyze urine metabolic profiles in term and preterm infants in order to reveal distinct metabolic patterns associated with different GA classes.

MATERIALS AND METHODS

3.1. Study population

Newborn infants admitted to the Neonatal Intensive Care Unit (NICU), University of Cagliari, Italy, or the Pediatric Division, Pescia Hospital, Italy, were prospectively enrolled and sampled throughout a 6-month period. Both preterm infants (GA of less than 37 weeks) and healthy full-term infants (GA of 37 to 41 weeks) with detectable urine output in the first 12 hours of life were eligible for this study. No exclusion criteria were applied to the preterm infant group. For each newborn infant, the major demographic and clinical data were abstracted from hospital records. This study did not involve any intervention apart from the usual medical procedures. Moreover, confidentiality and anonymity of the subjects was maintained, and sample collection followed institutional ethics guidelines.

3.2 Specimen collection and preparation

A random urine sample (volume: ≈2 mL) was collected non-invasively from each patient within the first 12 hours of life by means of a previously validated (17) and used (18-20) method, namely the cotton wool ball method. To minimize nutritional or xenobiotic influences on urinary biochemical composition, urine samples were collected shortly after birth. Each urine sample was centrifuged at 4°C and 13000 rpm for 5 min to remove insoluble material. An aliquot of 1 mL of supernatant was transferred to an Eppendorf tube prior to addition of 10 mul of a stock solution of NaN_3 used as a preservative (final concentration: 0.1 mM). The final sample was stored at -80°C until NMR analysis. A 400 mul aliquot of thawed urine was mixed with 200 mul of 0.2 M phosphate buffer solution (pH 7.4) to stabilise urinary pH. Subsequently, an aliquot of 50 mul of TSP (3-trimethylsilyl-$^2H_4$-propionic acid) in D_2O (1 mg/mL) was added to a final concentration of 0.8 mM to provide an internal reference for chemical shifts (0.00 ppm).

3.3 NMR spectroscopic analysis

All ^{1}H NMR spectra were acquired at 399.94 MHz on a Varian 400 Unity Inova spectrometer. Experiments were performed in 5-mm NMR tubes at 27.0 ± 0.1°C. The water signal was suppressed using the first increment of a NOESY pulse sequence with irradiation during a relaxation delay of 2 s as well as during a mixing time of 150 ms. Typically, 128 free induction decays (FIDs) were acquired using a spectral width of 6000 Hz, a 90° pulse of 6.4 mus, an acquisition time of 4 s, and a total pulse recycle time of 6 s. Prior to Fourier transformation, the FIDs were zero-filled to 132k and multiplied by an exponential weighting function corresponding to a line broadening of 0.3 Hz. Peak assignments were performed with the aid of literature data (21).

3.4 Data analysis

NMR spectra were normalized using the creatinine resonance. In order to enhance the use of all metabolic information in the NMR spectra according to a reproducible procedure, the number of spectral data points was reduced by segmenting the spectra into consecutive non-overlapping regions (buckets) and integrating the signal intensity in each region using a software program by Mnova (Mestrelab Research S.L.) (22). This reduction was achieved by the binning of data on regular 0.04 ppm width bins in the range of chemical shift region of interest: [10-5.5]-[4.5-0] ppm. The region of the water (4.5- 5-5.5 ppm) was not analyzed. The resulting normalized integrals or "buckets" were submitted to multivariate analysis by using SIMCA-P (Version 12, Umetrics, Umeå, Sweden) software package. Principal Component Analysis (PCA) models were initially built to identify outliers. This multivariate method is unsupervised. Subsequently, supervised Partial Least Squares (PLS) and PLS–Discriminant Analysis (PLS-DA) modelling was performed. PCA is a technique

Table 1. Demographic characteristics of the study population

Characteristic	***Term infants* (n=26)**	***Preterm infants* (n=41)**
Gender (M/F)	15/11	21/20
Gestational age (wk)*	39.7 ± 0.8	32.0 ± 3.0
Birth weight (Kg)*	3.3 ± 0.4	1.8 ± 0.8

*Results are expressed as mean ± standard deviation

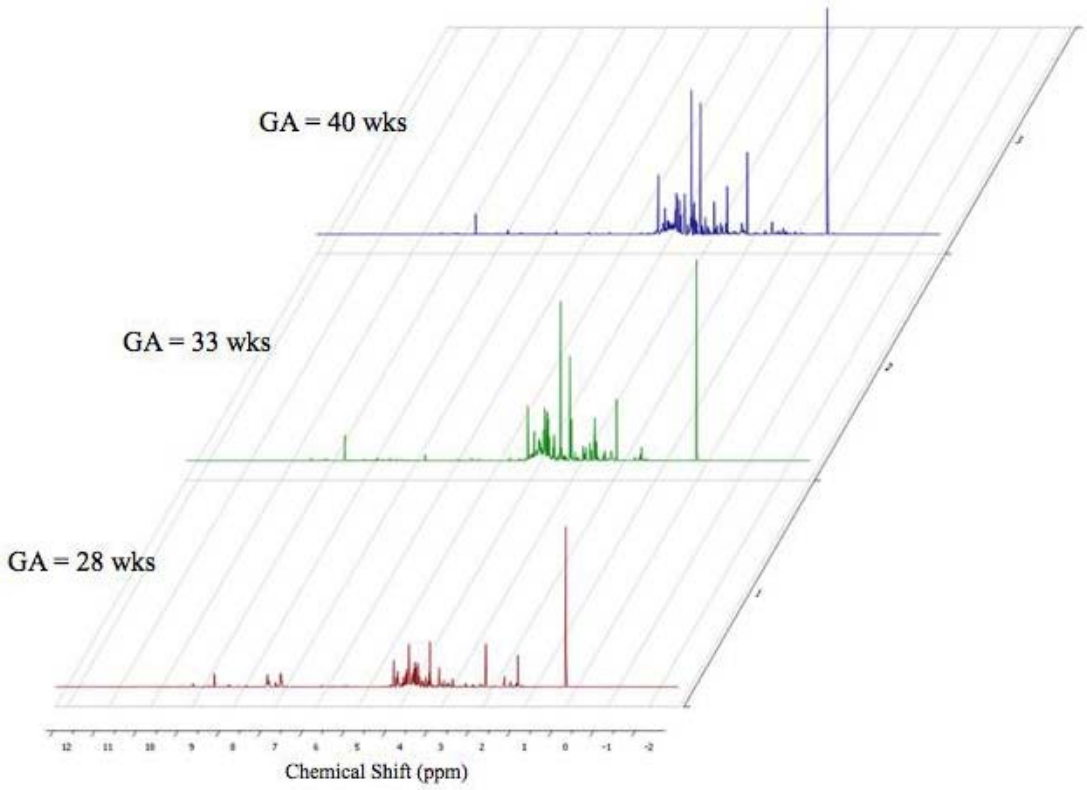

Figure 1. Representative urine ^{1}H-NMR spectra from a term infant, and two preterm infants of 33 and 28 weeks' gestation, respectively. Relevant profile differences may be noted examining the different spectral regions.

designed to express the information contained in an original set of highly correlated variables in a new set of uncorrelated variables, called principal components. PLS is a multivariate regression tool in which one or more Y-variables are regressed to the x-matrix (in this case the binned spectral data). PLS-DA is an extension optimized to provide information on class distinctions (e.g. different treatments or conditions) and Variable Importance in the Projection (VIP). Terms with VIP>1 have an above average influence on Y. The coefficients of goodness of fit (R^2) and prediction (Q^2) were used to assess the model quality. Finally, Hierarchical Cluster Analysis (HCA), a statistical method for finding relatively homogeneous clusters of cases based on measured characteristics, was performed by using SIMCA-P+ software.

4. RESULTS

A total of 67 newborns were enrolled in the study, 26 of whom were healthy full term infants and the remaining 41 were preterm infants. The major demographic characteristics of study population are summarized in Table 1. All patients were discharged home except one, born with a GA of 23 weeks, who died during hospitalization as a result of the complications of extreme prematurity (respiratory distress syndrome, and intraventricular hemorrhage). Assuming the metabolic state of healthy full term infants at birth to be the normal status, we hypothesized that the condition of prematurity might be associated with a range of urine NMR spectra reflecting different metabolic maturation states. All urine samples were analyzed with NMR technique to characterize specific metabolic profiles associated with prematurity and to identify the significant components leading to discrimination between the class of term infants and the class of preterm infants. Representative NMR urine spectra from a term infant, and two preterm infants of 33 and 28 weeks' gestation, respectively, are shown in Figure 1. With the aim of investigating the specific metabolic patterns within the data, the complete set of NMR spectra was analyzed using PCA, an unsupervised test which represents each individual spectrum as a single point in a scores plot (Figure 2). The specific location of each urine sample within the plot is defined by the loadings. In the PCA

Table 2. Demographic characteristics of groups A (23-32 weeks' gestation) and B (33-36 weeks' gestation) of preterm infants

Characteristic	***Group A* (n=22)**	***Group B* (n=19)**
Gender (M/F)	11/11	10/9
Gestational age (wk)*	30.0±2.6	34.4±1.3
Birth weight (Kg)*	1.4±0.6	2.4±0.5

*Results are expressed as mean ± standard deviation

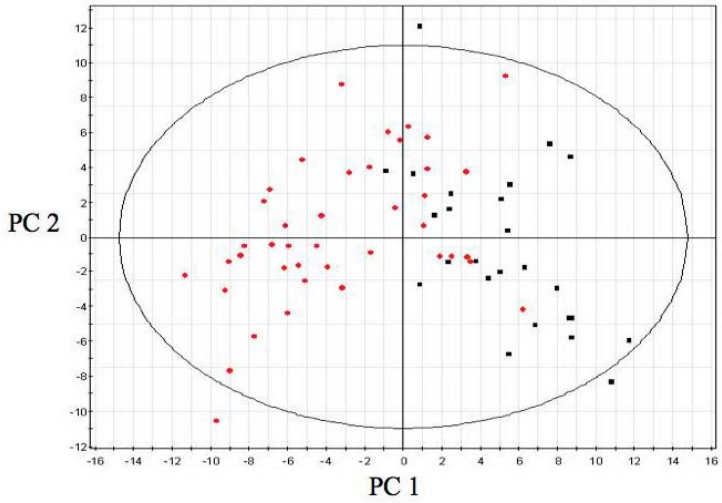

Figure 2. PCA scores plot (PC1 vs. PC2) generated in SIMCA-P from the full dataset. Urine samples are designated as follows: term infants, black squares; preterm infants, red diamonds. The map obtained using this unsupervised technique revealed two distinct clusters with an overlapping region.

cores plot, urine samples which are more similar cluster together. Data distribution revealed two clusters with overlapping regions (R^2X(cum): 0.661). For the purpose of the present study, preterm infants were arbitrarily divided into two groups according to GA: the first one (group A) included newborn infants of 23 to 32 weeks' gestation, while the second one (group B) included newborns of 33 to 36 weeks' gestation (Table 2). PCA analysis highlighted the difference in variance of NMR spectra obtained from preterm infants into two distinct classes according to the GA, as illustrated in the PCA scores plot (Figure 3). The results of PCA analysis encouraged further data processing in order to achieve a classification model for identifying the differences in the urinary metabolite profile. A PLS-DA based on the GA groups was performed and a model with a poor predictive power was calculated (R^2X (cum): 0.2; R^2Y (cum): 0.74; Q^2 (cum):0.21) (Fig.4). As an alternative to arbitrary class assignment based on GA, an unsupervised HCA procedure was applied, with the threshold in the dendrogram being set at the level of the separation of the two highest classes and a singlet. On the basis of classifications obtained, a supervised PLS-DA model was calculated; the score components are illustrated in Figure 5 (R^2X (cum): 0.26; R^2Y (cum): 0.97.; Q^2 (cum):0.79). Analysis of PLS-DA based on HCA generated one class of 13 samples and a second class including 27 samples. The first class consisted of 12 samples from preterm infants of GA 33-36 wks with minimal disorders, plus 1 sample from a preterm infant of GA 30 wks affected by respiratory distress syndrome, patent ductus arteriosus, and patent foramen ovale. The second class included 27 samples, 21 of which from preterm infants of GA 23-32 wks having a clinical condition consistent with a very low GA, 5 samples from preterm infants of GA 33-36 wks. The most important variables in the PLS-DA model, defined as VIPs reflect the importance of terms in the model both with respect to Y (i.e. its correlation to all the responses), and with respect to X. VIPs from this model were as follows: hippurate, tryptophan, phenylalanine, malate, tyrosine, X-hydroxybutyrate, N-acetyl-glutamate and proline. According to the information provided on the Kegg website on the metabolomic classification of biofluids (23), the most important metabolic cycles related to the variation of this set of metabolites are as follows: tyrosine metabolism; tyrosine, tryptophan, and phenylalanine biosynthesis; urea cycle; arginine and proline metabolisms.

5. DISCUSSION

Despite advances in the knowledge of risk factors and therapy, in developed countries the rate of preterm birth is increasing rather than decreasing (1). During the last decades, the survival rate of extremely preterm infants has increased, with a considerable increase in economic and social costs. Similarly, there has been a growing concern about the ethical issues surrounding the most premature infants who are at the limits of viability and intact survival. Currently, little is known about the overall metabolic status of term and preterm neonates, and only a limited number of metabolites are routinely measured in their biological fluids by conventional methods. The clinical management of preterm infants could probably be

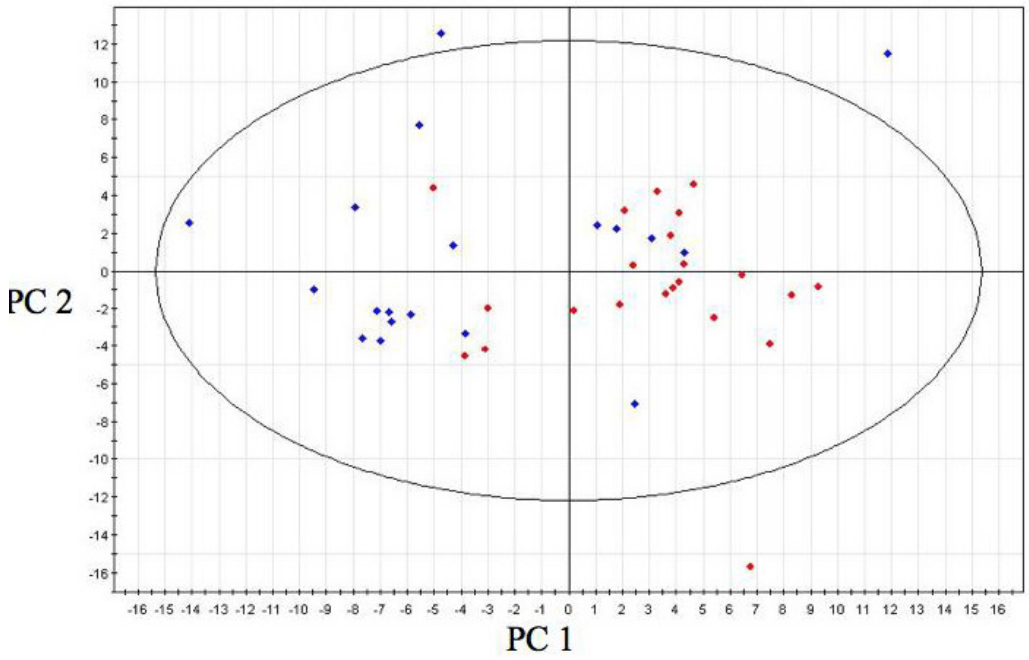

Figure 3. PCA scores plot (PC1 vs. PC2) generated in SIMCA-P from the pre-term samples. Urine samples are designated as follows: preterm infants of 23 to 32 weeks' gestation (group A), red diamonds; preterm infants of 33 to 36 weeks' gestation (group B), blue diamonds. The map generated using this unsupervised technique revealed two distinct clusters with a limited overlapping region.

improved if more information about perinatal and neonatal maturation processes and their metabolic background was available. It is already known that alterations in the metabolic profile at birth have been shown to be associated with adult pathological conditions (24,25). The identification of GA-related differences at birth in the urine metabolite profile is a requirement to investigate and monitor the metabolic status in term and preterm neonates. The main purpose of the present study was to identify distinct metabolic signatures capable of discriminating between different GA classes of neonates by using metabolomic analysis of urine. Metabolomic analysis is a holistic, non-invasive, and low cost approach particularly valuable for classification, prediction of physiopathological states (7, 26, 27), and identification of endogenous biomarkers of toxicity (28). The sensitivity of metabolomic technology relies on the ability to identify small variations in metabolic profiles associated with pathological conditions, or induced by environmental factors. High field proton nuclear magnetic resonance spectroscopy is a powerful technique that provides extensive information on both the structure and composition of low molecular weight metabolites in biological fluids. NMR analysis affords a number of advantages: it is characterized by a very high reproducibility, is non-selective with regard to metabolites undergoing detection, and requires no pre-selection of metabolites. Other advantages of this technology are the relatively short measurement time, the use of small sample volumes, the possibility to further investigate unaltered samples by conventional methods, and the economy of the single analysis. Conversely, disadvantages of NMR analysis include: relatively low sensitivity when applied to low-abundance molecular species, and the cost of initial equipment. ^{1}H NMR analysis of body fluids (especially urine, plasma, and cerebrospinal fluid) has become of value in the clinical diagnosis and monitoring of inherited metabolic diseases, and in the study of the biochemical environment of the fetus. The use of non-invasive techniques is an essential requirement in neonatal medicine, especially in very preterm infants in whom it may be difficult or traumatic to obtain even small samples of blood. In clinical settings, urine is routinely tested because it may provide meaningful diagnostic information and is collected by non-invasive methods. It is a complex biofluid constituted by numerous components, including by-products that reflect specific metabolic processes. Urine is widely used in metabolomic studies as a source of whole organism metabolic information. However, the composition of this biofluid may be also affected by changes in renal function, which should be taken into account when interpreting the results from this study. Prenatal and neonatal events, in combination with genetic factors, may influence renal development and function in the newborn. These factors have been found to play a crucial role in affecting nephrogenesis, the final complement of nephrons being critically dependent on GA and a favorable intrauterine environment (28-31). The histological study by Rodriguez *et al* (32) showed that the nephron number in premature infants was dramatically reduced, and that nephrogenesis could continue after preterm birth, but it did not progress beyond 40 days after delivery. Moreover, lower glomerular filtration rate and disturbed tubular function have been shown in school-age children born with

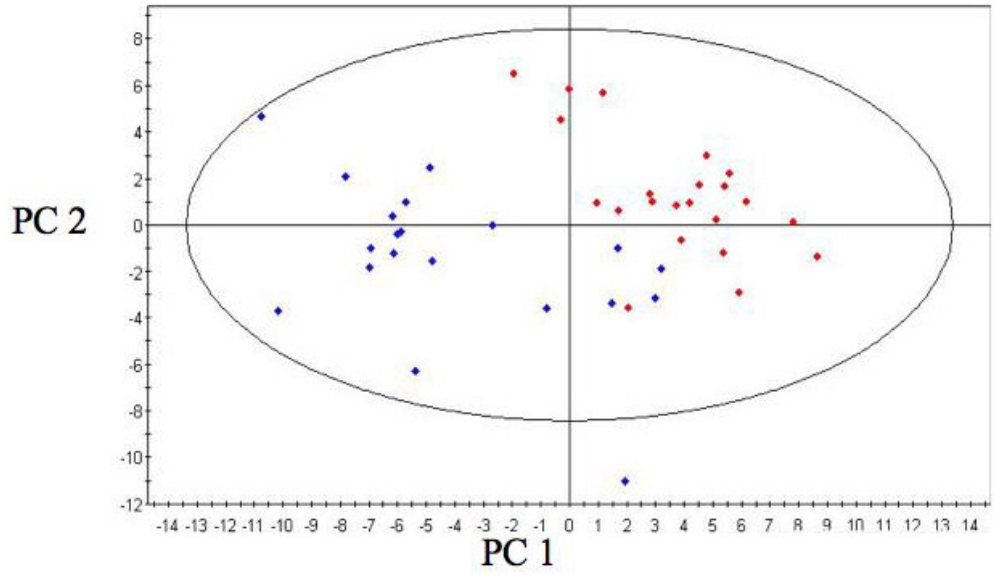

Figure 4. PLS-DA scores plot generated in SIMCA-P based on gestational age groups. Urine samples are designated as follows: preterm infants of 23 to 32 weeks' gestation (group A), red diamonds; preterm infants of 33 to 36 weeks' gestation (group B), blue diamonds. The map generated using this supervised technique revealed two clusters with overlapping region.

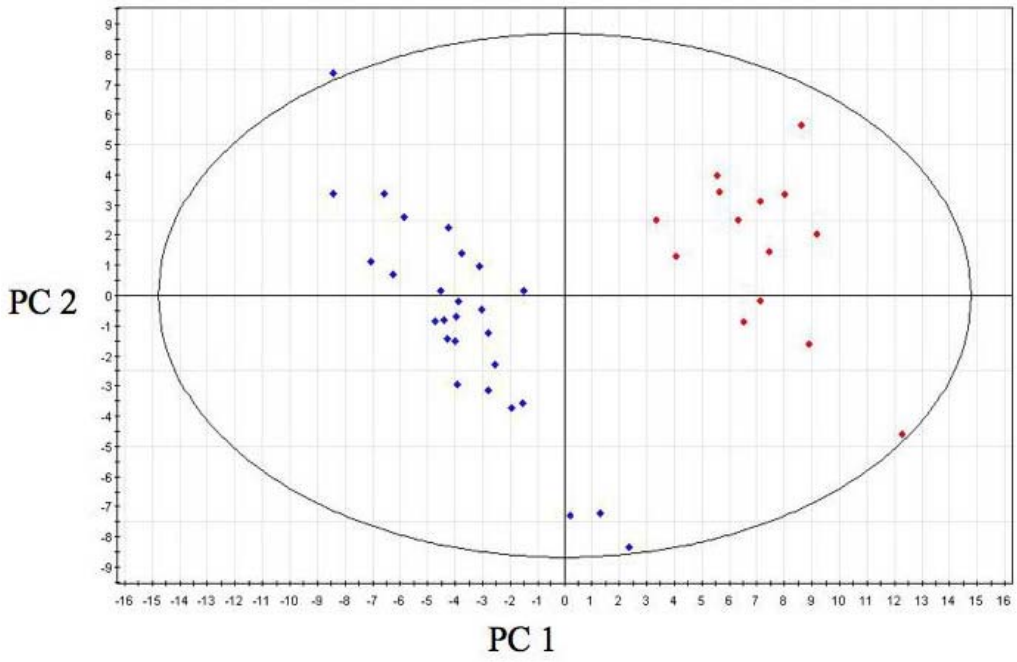

Figure 5. PLS-DA scores plot generated in SIMCA-P based on HCA. Blue diamonds are preterm infants with a "very immature" metabolic profile; red diamonds are preterm infants with an "immature " metabolic profile. The map generated using this supervised technique revealed two distinct clusters.

extreme prematurity (33). In our study population, ^{1}H NMR-based metabolomic analysis of urine clearly demonstrated that premature neonates featured a different metabolic profile compared to full-term neonates. In particular, multivariate analysis of urine spectral data revealed a clustered distribution with a significant separation of the term infant samples and preterm infant samples. Similarly, the urine samples from the two different GA classes of preterm infants showed a clustered distribution. Moreover, data analysis enabled the identification of the main discriminating low molecular weight metabolites, suggesting that amino acid biosynthesis and metabolism are the key metabolic mechanisms underlying fetal and perinatal maturation processes. Our

metabolomic approach was aimed at analyzing the metabolite composition of NMR urine spectra rather than the urinary concentrations of individual metabolites. In fact, the mere determination of the main urinary metabolites is poorly informative due to the numerous determinants affecting urine components. Therefore, in metabolomic-based studies, the availability of an adequate control group, and well-controlled experimental conditions are essential to the reliability of results. To define a "normal" neonatal metabolic status is a necessary condition for using a metabolomic approach in neonatal medicine. For the purpose of the present study, the urine metabolic pattern from healthy neonates born at term was considered as a "normal" pattern. In order to minimize the postnatal influences (diet, age, drugs, physical stimuli, etc) on metabolic processes, urine samples from all patients were collected within 12 hours of birth. Our findings should be interpreted with caution in the light of certain limitations of the study. In particular, our results may be biased by variables such as maternal pathological conditions, mode of delivery, perinatal and neonatal disorders, and iatrogenic factors, that were not analyzed in this study. Further large-scale prospective clinical studies using metabolomic approach are required in order to confirm our preliminary findings and investigate selected groups of neonates affected by specific disorders or under particular conditions.

6. CONCLUSIONS

The coupling of the ^{1}H-NMR analysis of urine with pattern recognition methodologies, in a population of term and preterm infants, was able to reveal distinct "metabolic" classes. These classes were not always associated with different GA classes, suggesting that the metabolic status of neonates at birth is not only dependent on their GA. In addition, pattern recognition analysis allowed the identification of the compounds underlying the different metabolic profiles in our study population.^{1}H-NMR metabolomic analysis of urine appears to be able to assess the metabolic status of preterm and term infants at birth. Other possible applications of metabolomic analysis of urine in newborns are the monitoring of postnatal metabolic maturation over time, the identification of biomarkers as early predictors of outcome and the implementation and monitoring of a tailored management of neonatal disorders. A close cooperation between basic and clinical scientists should be undertaken in an attempt to achieve a more widespread use of metabolomic techniques in perinatal and neonatal medicine.

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8. REFERENCES

1. Slattery MM, Morrison JJ. Preterm delivery. *Lancet* 360, 1489–97 (2002)

2.Hamilton BE, Martin JA, Ventura SJ. Births: preliminary data for 2006. *National Vital Statistics Reports* 56, 1-18 (2007)

3. McCormick MC. The contribution of low-birth weight to infant mortality and childhood morbidity. *N Engl J Med* 312, 82-90 (1985)

4. Kaddurah-Daouk R, Kristal BS, Weinshilboum RM. Metabolomics: a global biochemical approach to drug response and disease. *Annu Rev Pharmacol Toxicol* 48, 653-83 (2008)

5. Nicholson JK, Wilson ID. High resolution proton magnetic resonance spectroscopy of biological fluids. *Prog NMR Spectroscopy* 21, 449-501 (1989)

6. Villas-Bôas SG, Mas S, Åkesson M, Smedsgaard J, Nielsen J. Mass spectrometry in metabolome analysis. *Mass Spectrometry Reviews*. 24, 613-646 (2004)

7. Ellis DI, Dunn WB, Griffin JL, Allwood JW, Goodacre R. Metabolic fingerprinting as a diagnostic tool. *Pharmacogenomics*. 8, 1243-1266 (2007)

8. Holmes E, Tsang TM, Tabrizi SJ. The application of NMR-based metabonomics in neurological disordes. *NeuroRx* 3, 358-72 (2006)

9. Yang Y, Li C, Nie X, Feng X, Chen W, Yue Y, Tang H, Deng F. Metabonomic studies of human hepatocellular carcinoma using high-resolution magic-angle spinning (1)H NMR spectroscopy in conjunction with multivariate data analysis *J Proteome Res* 6, 2605-2614 (2007)

10. Videen JS, Ross BD. Proton nuclear magnetic resonance urinalysis: Coming of age. Kidney Int; 46, s122-128 (1994)

11. Holmes E, Foxall PJD,Nicholson JK, Neild GH, Brown SM, Beddell CR *et al.* Automatic data reduction and pattern recognition methods for analysis of 1h nuclear magnetic resonance spectra of human urine from normal and pathological states. *Analyt Biochem* 220, 284-296 (1994)

12. Atzori L, Antonucci R, Barberini L, Locci E, Cesare Marincola F, Scano P, Cortesi P, Agostiniani R, Weljie A, Lai A, Fanos V. 1H-NMR-based metabolic profiling of urine from children with nephrouropathies. *Front Biosci* E2,725-732 (2010)

13. Foxall PJD, Nicholson JK. Nuclear magnetic resonance spectroscopy: A non-invasive probe of kidney metabolism and function. *Exper Nephrol* 6, 409-14 (1998)

14. Brown JCC, Mills GA,SadlerPJ, Walker V. 1H NMR studies of urine from premature and sick babies. *Magn Reson Medicine* 11, 193-201 (1989)

15. Foxall PJD, Bewley S, Neild GH, Rodeck CH, Nicholson JK. Analysis of fetal and neonatal urine using

proton nuclear magnetic resonance spectroscopy. *Arch Dis Child* 73, F153-57 (1995)

16. Trump S, Laudi S, Unruh N, Goelz R, Leibfritz D. 1H-NMR metabolic profiling of human neonatal urine. *Magn Reson Mater Phy* 19, 305-12 (2006)

17. Fell JM, Thakkar H, Newmann DJ, Price CP. Measurement of albumin and low molecular weight proteins in the urine of newborn infants using a cotton wool ball collection method. *Acta Paediatr* 86, 518-22 (1997)

18. Cuzzolin L, Mangiarotti P, Fanos V. Urinary PGE(2) concentrations measured by a new EIA method in infants with urinary tract infections or renal malformations. *Prostaglandins Leukot Essent Fatty Acids* 64, 317-22 (2001)

19. Agostiniani R, Mariotti P, Cataldi L, Fanos V, Sani S, Zaccaron A, Cuzzolin L. Role of renal PGE2 in the adaptation from foetal to extrauterine life in term and preterm infants. *Prostaglandins Leukot Essent Fatty Acids*. 67, 373-77 (2002)

20.Antonucci R, Cuzzolin L, Arceri A, Dessì A, Fanos V. Changes in urinary PGE2 after ibuprofen treatment in preterm infants with patent ductus arteriosus. *Eur J Clin Pharmacol.* 65, 223-30 (2009)

21. Lundberg, P., Vogel, T., Malusek, A., Lundquist P.-O., Cohen, L., Dahlqvist, O. MDL -*The Magnetic Resonance Metabolomics Database (mdl.imv.liu.se),* ESMRMB, Basel, Switzerland. (2005)

22. www.mestrelab.com

23. http://www.genome.jp/kegg/brite.html

24.Stockor JP, Holmes E. Metabonomics applications in toxicity screening and disease diagnosis. *Curr Topics Medic Chem* 2, 35-51 (2002).

25. Moolenaar S, Engelke UF, Wevers RA. Proton nuclear magnetic resonance spectroscopy of body fluids in the field of inborn errors of metabolism. *Ann. Clinic Biochem* 40, 16-24 (2003)

26. Griffin JL, Nicholls AW. Metabolomics as a functional genomic tool for understanding lipid dysfunction in diabetes, obesity and related disorders. *Pharmacogenomics* 7, 1095-107 (2006)

27. Griffin JL, Kauppinen RA. A metabolomics perspective of human brain tumours. *FEBS J* 274**,** 1132-1139 (2007)

28. Coen M, Holmes E, Lindon JC, Nicholson JK. N MR-based metabolic profiling and metabonomic approaches to problems in molecular toxicology. *Chem Res Toxicol* 21, 9-27 (2008)

29. Hoy WE, Hughson MD, Bertram JF, Douglas-Denton R, Amann K. Nephron number, hypertension, renal disease, andrenal failure. *J Am Soc Nephrol* 16, 2557-64 (2005)

30. Hughson M, Farris AB, Douglas-Denton R, Hoy WE, Bertram JF. Glomeral number and size in autopsy kidney: The relationship to birth weight. *Kidney Int* 63, 2113-22 (2003)

31. Puddu M, Fanos V, Podda F, Zaffanello M. The kidney from prenatal to adult life: Perinatal programming and reduction of number of nephrons during development. *Am J Nephrol* 30, 162-170 (2009)

32. Rodríguez MM, Gómez AH, Abitbol CL, Chandar JJ, Duara S, Zilleruelo GE. Histomorphometric analysis of postnatal glomerulogenesis in extremely preterm infants. *Pediatr Dev Pathol* 7, 17-25 (2004)

33. Rodríguez-Soriano J, Aguirre M, Oliveros R, Vallo A. Long-term renal follow-up of extremely low birth weight infants. Pediatr Nephrol 20:579-84 (2005)

Abbreviations: NMR: Nuclear Magnetic Resonance

Key Words: Metabolomics, Preterm Neonates, Non Invasive Analysis

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