[Frontiers in Bioscience E2, 725-732, January 1, 2010]

^{1}H NMR-based metabolic profiling of urine from children with nephrouropathies

Luigi Atzori[1], Roberto Antonucci[2], Luigi Barberini[3], Emanuela Locci[4], Flaminia Cesare Marincola[4], Paola Scano[4], Patrizia Cortesi[5], Rino Agostiniani[5], Aalim Weljie[6], Adolfo Lai[4], Vassilios Fanos[2]

[1]Department of Toxicology, University of Cagliari, Italy,[2]Department of Pediatrics and Clinical Medicine-Section of Neonatal Intensive Care Unit, University of Cagliari, Italy, [3]Department of Neurological Sciences, University of Cagliari, Italy, [4]Department of Chemical Sciences, University of Cagliari, Italy, [5]Pediatric Division, Pescia Hospital, Italy, [6]Department of Biological Sciences, University of Calgary, Canada

TABLE OF CONTENTS

1. ABSTRACT

Pediatric nephrourological diseases are associated with functional alterations frequently related to inflammatory states. A feedback loop adjusts urinary system function while forcing adaptation to internal and external influences during disease development and as a result of treatment. We hypothesized that nephrourological dysfunction would alter the urine metabolite pattern in children in a defined manner. To characterize the metabolite patterns associated with nephrouropathies, a proton nuclear magnetic resonance (^{1}H NMR)-based metabonomic analysis was performed on urine samples obtained from twenty-one children affected by nephrouropathies and 19 healthy controls. Urine samples were analyzed with a 400 MHz Varian spectrometer and multivariate statistical techniques were applied for data interpretation. Linear discriminant analysis–based classification of the spectral data demonstrated high accuracy (95%) in the separation of the two groups of samples. By extension, the urine metabolite profiles were shown to correlate with nephrourological disorders in our model. In conclusion, ^{1}H NMR-based metabonomic analysis of urine appears to be a promising, non-invasive approach for investigating and monitoring pediatric nephrourological diseases.

2. INTRODUCTION

In clinical settings, biofluid analysis (e.g. blood, urine, cerebrospinal fluid, saliva) is used extensively to evaluate pathophysiological conditions, since the composition of biological fluids may reflect underlying metabolic processes. Urine has historically been examined for signs of illnesses, thus providing a fingerprint for different conditions. Compared to other biofluids, urine is readily accessible for analysis owing to its easy and non-invasive collection and might also provide information about metabolic changes related to different physiological and pathological status. Recent "omics" technologies consist of approaches with a holistic perspective on macromolecules such as genes and proteins: genomics and proteomics. In many contexts, genomics and proteomics provide limited evidence of endpoint markers in diagnosis, toxicology, pharmacology, and/or prevention. In contrast, this aspect may be satisfied by the youngest of the "omics" disciplines: metabonomics. Metabonomics defined as "the quantitative measurements of the dynamic multy-parametric response of living systems to to pathophysiological stimuli or genetic modifications" (1) is the comprehensive and simultaneous systematic determination of metabolite levels that are present within a cell, tissue organism in response to genetic modifications,

physiological conditions, pathological stimuli, drugs, diet, lifestyle via analysis of biofluids and tissues. This technique is based on the analysis of multi-parametric metabolic profiles mainly generated by NMR spectroscopy and/or mass spectrometry. This approach typically produces a large number of extremely complex datasets as a result of different conditions in biological systems. Multivariate spectroscopic data are often analysed using chemometric and pattern recognition techniques to extract latent metabolic information and enable sample classification. From these profiles it is possible to generate "metabolic profiles" associated to a particular set of pathways or class of compounds, or obtain "metabolite fingerprints". Metabonomics approaches applied to urine have identified differences due to gender (2), diurnal variation (3), and drug or toxins exposure (4). In recent years there has been increased application of NMR-based metabonomics in clinical settings and in disease diagnosis, e.g. neurological disorders (5), and cancer (6). Metabonomic analysis has been used to evaluate individual drug effects and predict possible toxicity and underlying mechanisms (7). Metabolic changes may predicted individual response to treatment prior to morphological damage (8). Paediatric studies have indicated that metabolic profiles may be useful in assessing the biological age (9), correlate with airway dysfunction in an asthma model (10), and identify early biomarkers of acute kidney injury after cardiac surgery (11). So, all together, information generated by a metabonomic approach may lead to an early diagnosis of disease, identification of new markers and development of a better personalized medicine. The fact that these analyses are not invasive is an important factor especially in children. Application of urine metabonomic analysis in newborn children could serve to monitor metabolic maturation over time, identify biomarkers as early predictors of outcome, and implement a tailored management of neonatal and paediatric disorders. We describe here the application of ^{1}H-NMR spectroscopy-based metabonomics to investigate urinary metabolic profiles, with the aim of generating new information about metabolic patterns associated with different nephrourological disorders in children.

3. MATERIALS AND METHODS

3.1. Subjects

The study was performed on two groups of children admitted to the Paediatrics Division, Pescia Italy. Twenty-one patients were affected by different nephrouropathies [renal dysplasia (n=5), vesico-ureteral reflux (n=7), urinary tract infection (n=4), acute kidney injury (n=2), and others (n=3)], and there were19 healthy control subjects. Clinical data from each patient was collected from hospital records. An urine sample (1 mL) was collected non-invasively from each patient using a standard protocol during hospitalization and then were stored at -80°C until NMR analysis. Urine samples were collected and prepared for the ^{1}H-NMR analysis with a 400 MHz Varian spectrometer.

3.2. Preparation of urine samples

A 400 mul aliquot of thawed urine was mixed with 200 mul of 0.2 M phosphate buffer solution (pH 7.4) to stabilize the pH of the urine. Then, an aliquot of 50 mul of TSP (3-trimethylsilyl-$^{2}H_4$-propionic acid) in D_2O was added to a final concentration of 0.8 mM to provide an internal reference for the chemical shifts (0 ppm).

3.3. NMR spectroscopy

^{1}H NMR spectra were acquired at 399.94 MHz on a Varian 400 Unity Inova spectrometer. The experiments were performed in 5-mm NMR tubes at 27°C. The water signal was suppressed using the first increment of a NOESY pulse sequence with irradiation during a relaxation delay of 2 s and also during a mixing time of 150 ms. Typically, 128 free induction decays (FID)s were collected 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.

3.4. Data reduction of the NMR spectra

In order to maximize the probability of using all the metabolic information in the spectra and to use a reproducible procedure, the spectra were reduced by segmentation into consecutive non-overlapped regions (buckets), and the spectral area in each region was integrated using the software program Mnova (Mestrelab Research S.L., www.mestrelab.com). Dimensionality reduction of the data matrix was achieved by binning the data into regular 0.04 ppm width regions across the chemical shift region of interest: [10-5.5]-[4.5-0] ppm. The resulting normalized integrals or "buckets" were subject to multivariate analysis using the SIMCA-P+ (Version 12.0, Umetrics, Umeå, Sweden) software package. Principal component analysis models were initially built to look for outliers, and subsequently supervised partial least squares (PLS), PLS–discriminant analysis (PLS-DA) or orthogonal PLS (OPLS) modelling was performed. PLS is a multivariate regression tool in which one or more Y-variables are regressed to the x-matrix. PLS-DA is an extension which is optimized to provide information about class distinctions (for example different treatments or conditions). OPLS performs an orthogonalization such that the variance with respect to Y is directly related to the primary component of the model in X.

4. RESULTS

In the present study, nineteen healthy children (11 boys and 8 girls; mean age +/- standard deviation, 7.2 +/- 4.3 years) and twenty-one children affected by nephrourological diseases (10 boys and 11 girls; mean age +/- standard deviation, 5.4 +/- 3.3 years) were recruited. Assuming the metabolic state of healthy children to be normal, we hypothesized that renal disorders could affect the metabolic profile of urine. All urine samples were analysed by NMR spectroscopy in order to evaluate differential metabolic profiles. Representative NMR urine spectra from a normal child and a child suffering from a renal disorder are shown in Figure 1A and Figure 1B. In order to characterize the multivariate structure in the data, an unsupervised Principal Component Analysis (PCA) model was created (Figure 2). This model showed some difference between the disease and control groups, which encouraged further analysis using supervised methods.

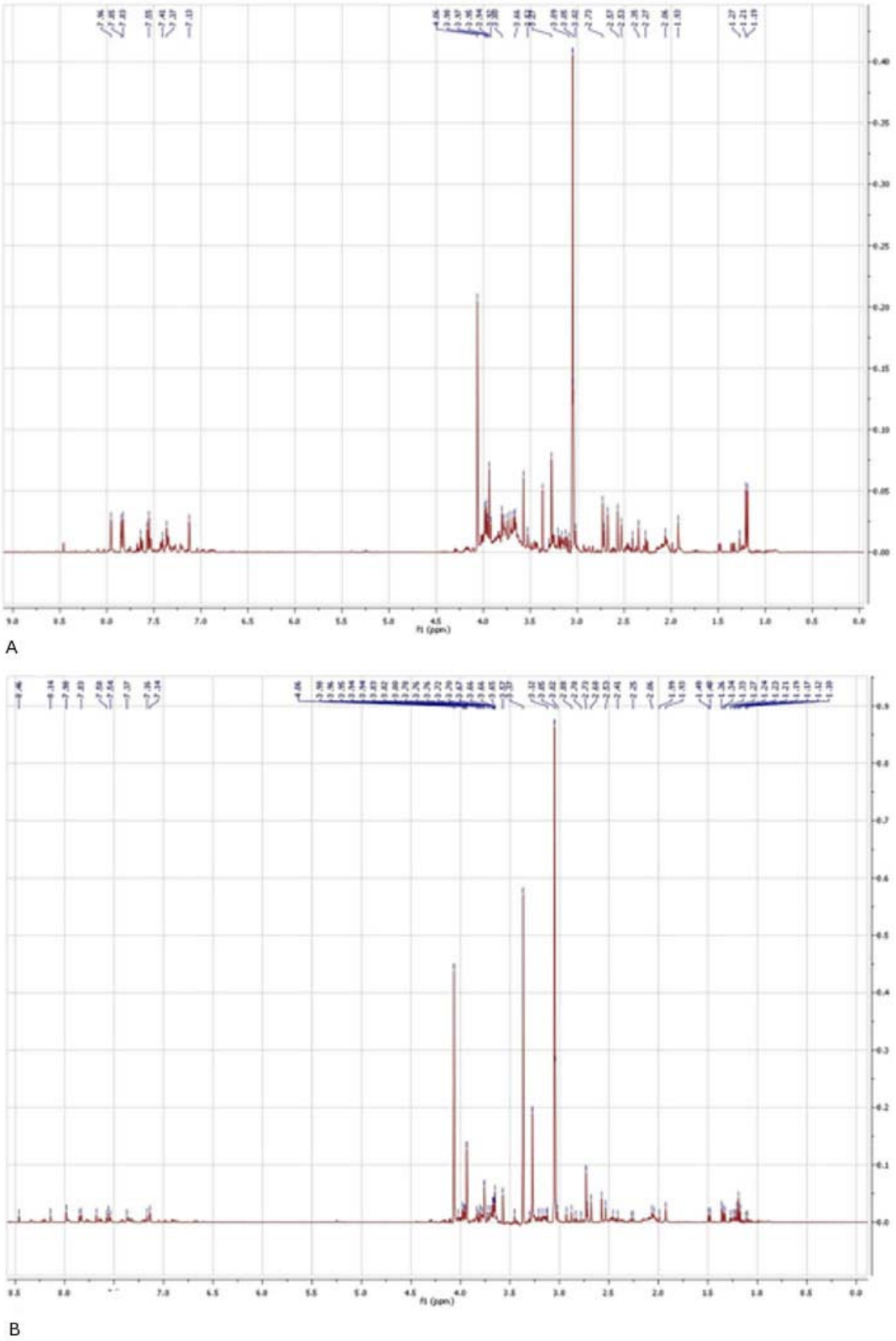

Figure 1. A) A 400 MHz ^{1}H-NMR spectrum of urine showing the metabolite profile characteristic of a healthy child. B) A 400 MHz ^{1}H-NMR spectrum of urine demonstrating the metabolite profile characteristic of a child affected by a nephrourological disease.

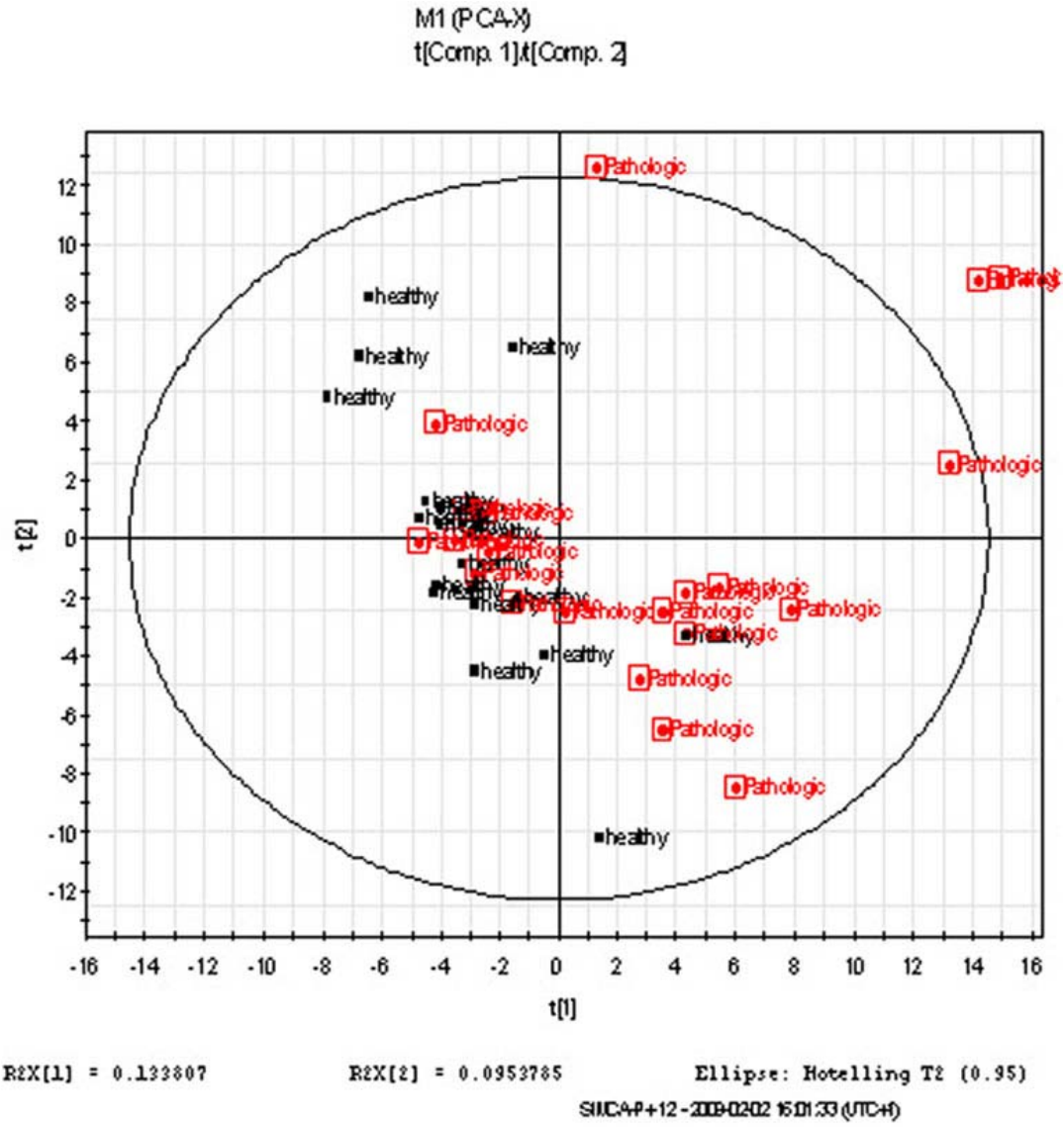

Figure 2. PCA scores plot of urinary spectral data of healthy vs. pathological children. The position of each point represents the combined metabolite profile of a single patient sample.

Partial least-squares discriminant analysis (PLS-DA) is a supervised extension of PCA which allows for class separation and interpretation of changes in the underlying metabolites.. The classification of different pathological conditions was performed using two different states (disease and control) as a preliminary model to discriminate metabolic profiles. In order to reduce the impact of variance in the x-matrix (NMR data) unrelated to the two groups ("noise" in the data), we also performed a supervised analysis based on the OPLS-DA algorithm implemented in SIMCA Software to perform an orthogonalization such that the variance with respect to Y is directly related to the primary component of the model in X (Figure 3). The misclassification list for OPLS/O2PLS-DA, that shows the proportion of correct classification of the workset, gave a total value of 95% (Fisher Value $3e^{-10}$).

In this model, we could identify the most important variable influence on projection (VIP) for the metabolites responsible for separation of the two groups. This data is illustrated in Figure 4. In our paediatric patients, we were able to identify some regions of interest in the chemical shift diagram of the urine metabolites that were possibly related to nephrouropathies. In fact, spectral alterations in the [3.5-3.9], [4,1-4.4], [8.2-8.6] chemical shift regions (Figure 5) are generated by renal cortex pathology strictly related to the alterations of purine and pyridine and to the alterations of the urea cycle (9, 12). Due to the fluxes in the metabolic cycles of nitrogen, the alterations we found, having "markers" in the 3.0-3.9 ppm region, could be considered as an important factor in the urea cycle and metabolism of amino groups. It is important to note that certain alterations found in this region of chemical shift of

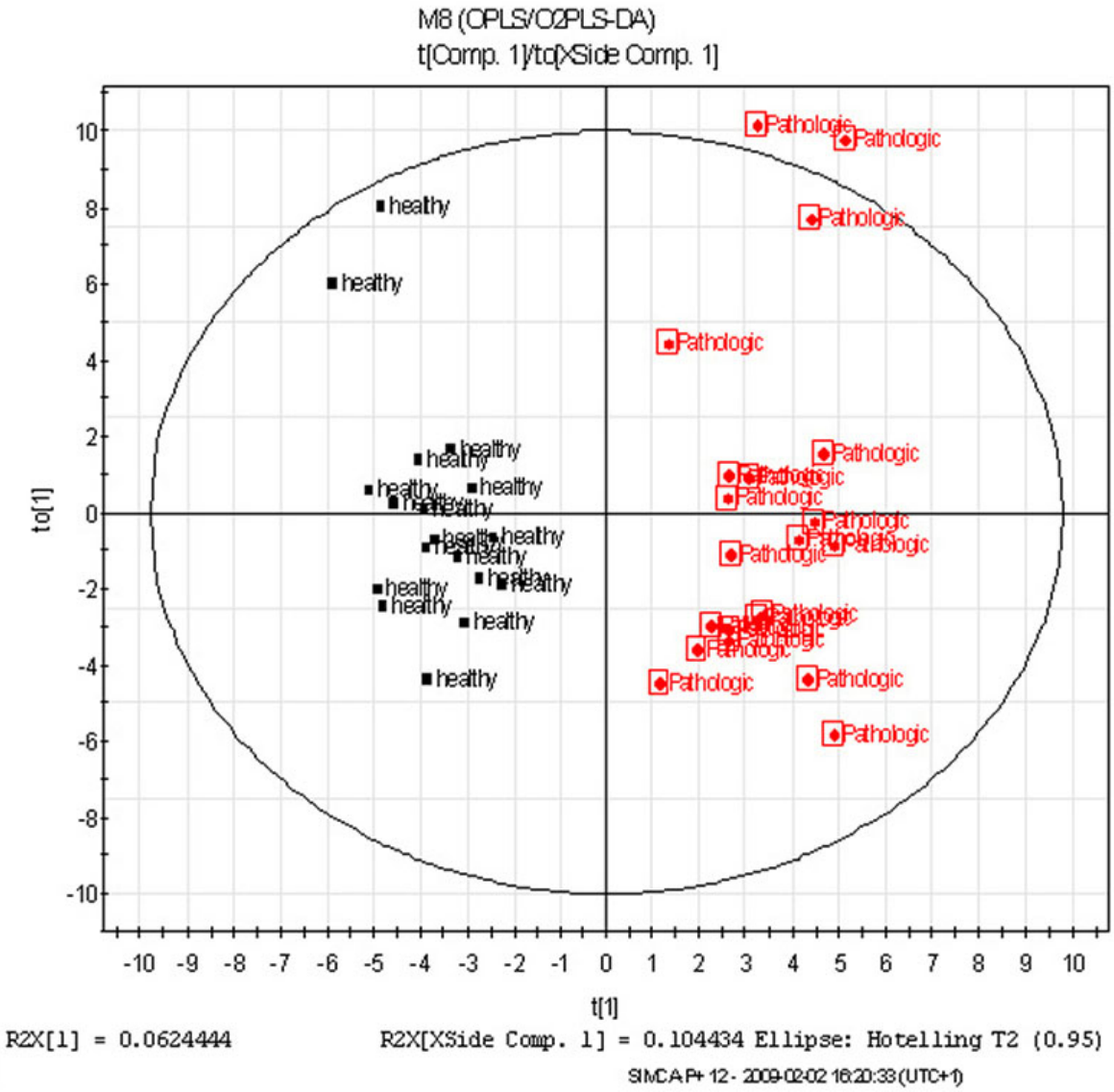

Figure 3. OPLS-DA scores plot derived from urinary spectral data of healthy vs. pathological children.

urine NMR spectra and connected with urea cycle are possibly related to alterations in the development and growth of children.

5. DISCUSSION

The aim of this study was to verify if a metabolic signature could discriminate certain pathological conditions affecting the kidney and/or urogenital tract. Urine NMR analysis enables one to quantify a large number of compounds and thus to give a relatively complete picture of metabolic mechanisms. In clinical settings, urine is routinely examined because of its non-invasive and easy collection. Urine is a complex biofluid containing several components, which include many by-products reflecting different metabolic processes. For this reason, this biofluid is largely used in metabonomic studies as a source of metabolic information for the whole organism. Metabolite changes can be associated with pathological conditions (13,14). Metabonomic approaches are non-invasive, holistic, and low cost analyses. The present study was aimed at analysing the metabolite composition of NMR urine spectra rather than the urinary concentrations of individual metabolites, since the mere determination of the main urinary metabolites is poorly informative. Moreover, sample variability is an important factor to consider in metabonomic studies of urine (15). High field proton nuclear magnetic resonance spectroscopy (^{1}H NMR) is a powerful, non-invasive, technique which provides extensive information on both the structure and the composition of low molecular weight metabolites in biological fluids. Human biofluids give characteristic ^{1}H NMR "fingerprints" of metabolites that are affected by the basic mechanism, severity, and location of a pathologic

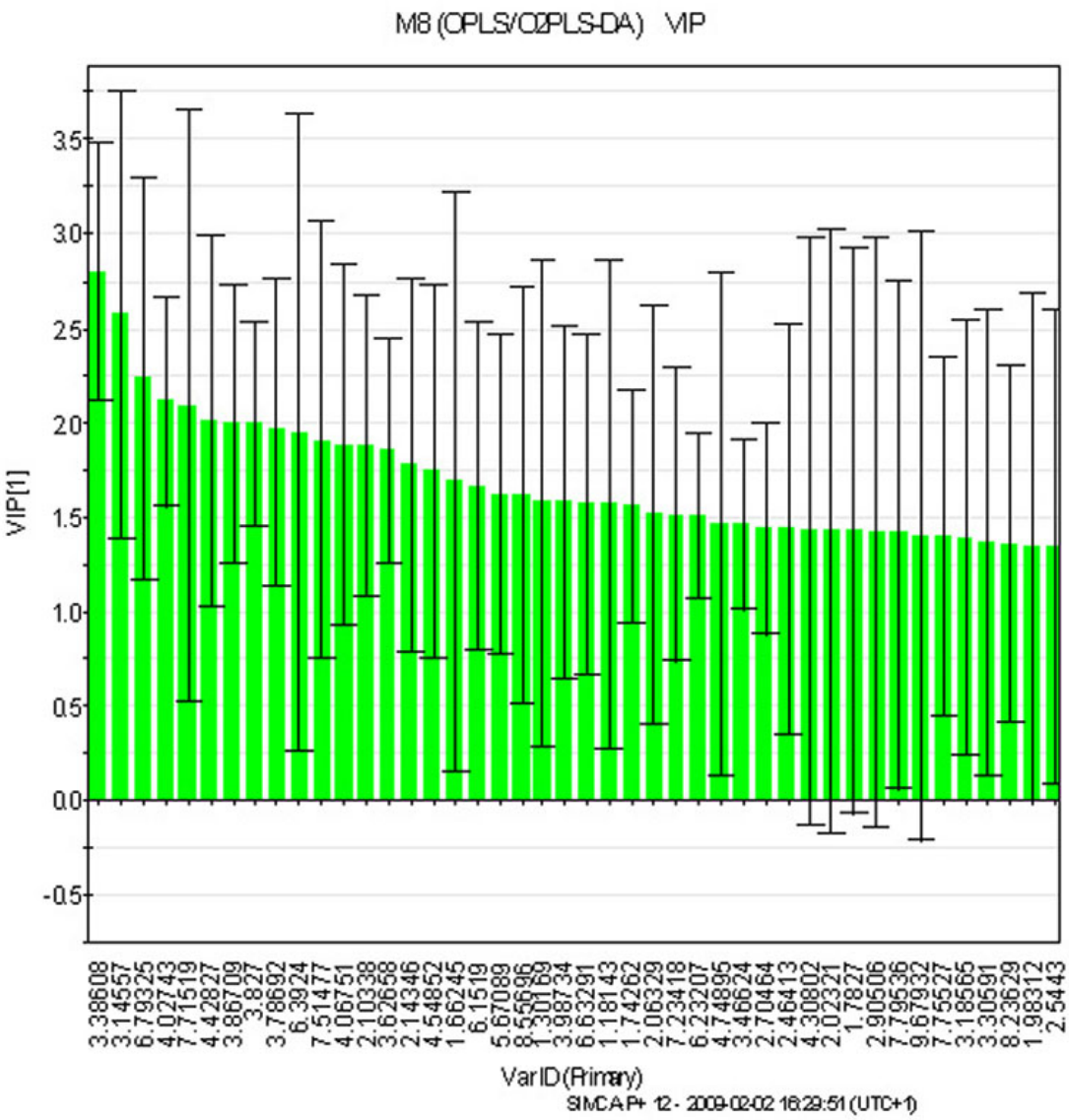

Figure 4. The VIP (variable influence on projection) plot summarizing the importance of the variables to explain X variances. VIP plots are sorted by order of importance and the ppm range can be correlated to the NMR signatures of individual metabolites.

lesion (16-21). Analysis of urine by NMR spectroscopy offers different advantages such as the following: a) non-destructive and non-invasive; b) wide range of metabolites detectable, and no requirement for metabolite pre-selection; b) small sample volumes required (typically 0.3-0.5 ml); c) rapid acquisition of NMR data (less than 10 minutes); d) very high reproducibility; e) low cost per sample. The sensitivity of "-omics" technologies, including metabonomics relies on the ability to identify small differences in pathological conditions. For this reason, in order to use a metabonomic approach it is essential to characterize the "normal" status, and in our study, we assumed that the "normal" urine pattern was that observed in healthy children. The analysis of urine by NMR does not require sample pre-treatment and the collection of the sample is non-invasive. In the future, the technique used to evaluate the variability in metabonomic data might have important applications in diagnosing metabolic diseases, evaluating drug response, monitoring therapy, or predicting the long-term evolution of renal disorders. In summary, urine ^{1}H-NMR metabonomic analysis is able to investigate the metabolic function of different organ systems, namely the kidney, and thus may be a useful tool for studying metabolic development in childhood. In fact, this analysis is able to detect a wide range of compounds in a small urine sample, avoiding the use of more complex methods.

6.CONCLUSIONS

In our model, alterations of urine ^{1}H-NMR spectra metabolites correlated with renal pathology. Urine NMR analysis appears to be a promising, non-invasive

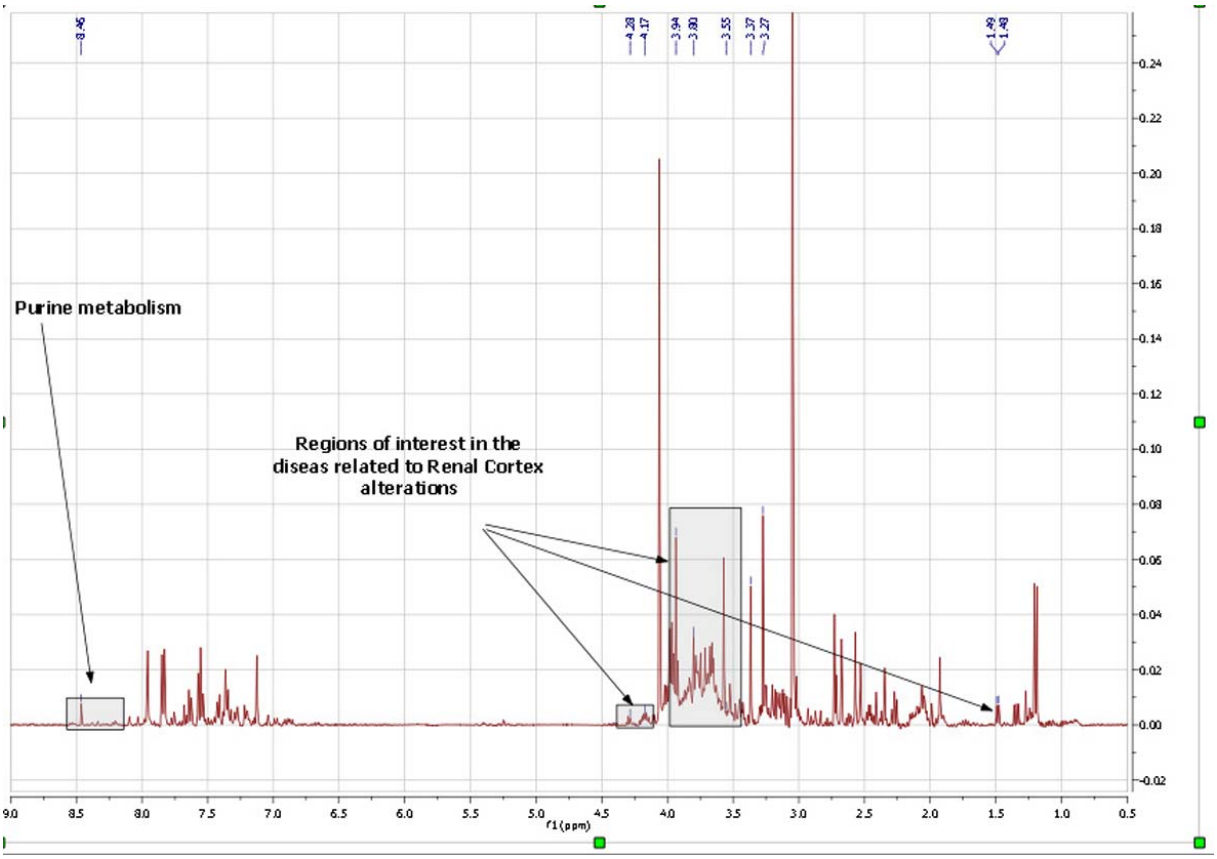

Figure 5. Regions of interest in the ^{1}H-NMR spectrum for nephrourological diseases.

technique for classifying and monitoring nephrourological disorders in paediatric patients.

7. ACKNOWLEDGEMENTS

We would like to thank the students Milena Lussu and Federica Murgia for their kind cooperation in the NMR analysis.

8. REFERENCES

1. Jeremy K Nicholson, John C Lindon, Elaine Holmes: "Metabonomics":understanding the metabolic response of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. *Xenobiotica* 29, 1181-1189 (1999)

2. Mark P Hodson, Gordon J Dear, Andy D Roberts, Claire L Haylock, Rachel J Ball, Robert S Plumb, Chris L Stumpf, Julian L Griffin, John N Haselden: A gender-specific discriminator in Sprague-Dawley rat urine: The deployment of a metabolic profiling strategy for biomarker discovery and identification. *Anal Biochem* 362,182-192 (2007)

3. Bollard ME, Holmes E, Lindon JC, Mitchell SC, Branstetter D, Zhang W, Nicholson JK: Investigations into biochemical changes due to diurnal variation and estrous cycle in female rats using high-resolution ^{1}H NMR spectroscopy of urine and pattern recognition. *Anal Biochem* 295, 194-202 (2001)

4. Muireann Coen, Elaine Holmes, John C Lindon, Jeremy K Nicholson: NMR-based metabolic profiling and metabonomic approaches to problems in molecular toxicology. *Chem Res Toxicol* 21, 9-27 (2008)

5. Elaine Holmes, Tsz M Tsang, Sarah J Tabrizi: The application of NMR-based metabonomics in neurological disorders. *NeuroRx* 3, 358-372 (2006)

6. Yongxia Yang, Chenglong Li, Xiu Nie, Xiansong Feng, Wenxue Chen, Yong Yue, Huiru Tang, Feng Deng: 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)

7. Rima Kaddurah-Daouk, Bruce S. Kristal, Richard M.Weinshilboum: Metabolomics: A Global Biochemical Approach to Drug Response and Disease. *Annu. Rev. Pharmacol. Toxicol* 48, 653–83 (2008)

8. T. Andrew Clayton, John C. Lindon, Olivier Cloarec, Henrik Antti, Claude Charuel, Gilles Hanton,Jean-Pierre Provost, Jean-Loı¨c Le Net, David Baker, Rosalind J. Walley, Jeremy R. Everett, Jeremy K. Nicholson:

Pharmaco-metabonomic phenotyping and personalized drug treatment. *Nature* 440: 1073-77(2006)

9. Haiwei Gua, Zhengzheng Panb, Bowei Xic, Bryan E. Hainlined, Narasimhamurthy Shanaiahb, Vincent Asiagob, G. A. Nagana Gowdab, Daniel Raftery: 1H NMR metabolomics study of age profiling in children. *NMR Biomed* (2009 May 13,Epub ahead of print)

10. Silvia Carraro, Serge Rezzi, Fabiano Reniero, Ka´roly He´berger, Giuseppe Giordano, Stefania Zanconato, Claude Guillou, and Eugenio Baraldi: Metabolomics Applied to Exhaled Breath Condensate in Childhood Asthma. *Am J Respir Crit Care Med* 175, 986–990 (2007)

11. Richard Beger, Ricky Holland, Jinchun Sun, Laura K. Schnackenberg, Page C. Moore, Catherine L. Dent, Prasad Devarajan and Didier Portilla: Metabonomics of acute kidney injury in children after cardiac surgery. *Pediatr Nephrol* 23, 977-984 (2008)

12. Jeremy K. Nicholson, John Connelly, John C. Lindon and Elaine Holmes: Metabonomics: a platform for studying drug toxicity and gene function. *Nature Rev Drug Discovery* 1, 153-161(2002)

13. John P Stockor, Elaine Holmes: Metabonomics applications in toxicity screening and disease diagnosis. *Curr Topics Medic Chem* 2, 35-51 (2002)

14.Sytske Moolenaar, Udo FH Engelke, Ron A Wevers: Proton nuclear magnetic resonance spectroscopy of body fluids in the field of inborn errors of metabolism. *Ann Clinic Biochem* 40, 16-24 (2003)

15. Erik J Saude, Darryl Adamko, Brian H Rowe, Tom Marrie T, Brian D Sykes: Variation of metabolites in normal human urine. *Metabolomics* 3, 439-451 (2007)

16. Jeremy K Nicholson, Ian D Wilson: High resolution proton magnetic resonance spectroscopy of biological fluids. *Prog NMR Spectroscopy* 21, 449-501 (1989)

17. John S Videen, Brian D Ross. Proton nuclear magnetic resonance urinalysis: Coming of age. *Kidney Int* 46, s122-128 (1994)

18. Elaine Holmes, Peta JD Foxall, Jeremy K Nicholson, Guy H Neild., Sarah M Brown, Christopher R Beddell, Brian C Sweatman, Elizabeth Rahr, John C Lindon., Manfred Spraul and Peter Neidig: Automatic data reduction and pattern recognition methods for analysis of 1h nuclear magnetic resonance spectra of human urine from normal and pathological states. *Anal Biochem* 220, 284-296 (1994)

19. Richard A Iles, Ronald A Chalmers: Nuclear magnetic resonance spectroscopy in the study of inborn errors of metabolism. *Clin Sci* 74, 1-10 (1988)

20. Peta JD Foxall, Jeremy K Nicholson: Nuclear magnetic resonance spectroscopy: A non-invasive probe of kidney metabolism and function. *Exper Nephrol* 6, 409-414 (1998)

21. Julian L Griffin, Risto A Kauppinen: A metabolomics perspective of human brain tumours. *FEBS J* 274, 1132-9 (2007)

Abbreviations: NMR: Nuclear Magnetic Resonance

Key Words: Metabonomics, Nephrourological Diseases, Non Invasive Analysis

Send correspondence to: Luigi Atzori, Dept of Toxicology, University of Cagliari, Italy, Tel: 390706758390, Fax: 39070666062, E-mail: latzori@unica.it

http://www.bioscience.org/current/vol2E.htm