

- 9 Abrams JT, Balin BJ, Vonderheid EC. Association between Sézary T cell-activating factor, *Chlamydia pneumoniae*, and cutaneous T cell lymphoma. *Ann N Y Acad Sci* 2001; **941**:69–85.
- 10 Koivisto AL, Isoaho R, Von Hertzen L et al. Chlamydial antibodies in an elderly Finnish population. *Scand J Infect Dis* 1999; **31**:135–9.

Funding sources: this work was supported in part by grants from Associazione Italiana per la Ricerca sul Cancro (to R.D., contract 10301). E.P. is a fellow of the Fondazione Italiana per la Ricerca sul Cancro.

Conflicts of interest: none declared.

An exploratory ^1H -nuclear magnetic resonance metabolomics study reveals altered urine spectral profiles in infants with atopic dermatitis

DOI: 10.1111/j.1365-2133.2011.10711.x

MADAM, Atopic dermatitis (AD) is a common chronic inflammatory skin disease in children. The prevalence of diagnosed AD in a population of preschool children has been observed to be above 15%,¹ therefore representing a major health impact in paediatrics. The pathogenesis of AD is only partially known,²

and little information is available about potential biomarkers. These facts make an easy and noninvasive test for evaluation of infant AD greatly desired by healthcare workers and parents.

Metabolomics provides a new approach and opportunity to explore the metabolic effects of many conditions in individuals,^{3,4} and recent evidence has demonstrated that it can be usefully employed in the characterization of airway biochemical fingerprints in children with allergic asthma.⁵ Nuclear magnetic resonance (NMR) spectra of biofluids contain signals from hundreds of metabolites. Chemometric approaches, such as principal component analysis (PCA) or cluster analyses, can be used to map the spectra in multivariate space where each sample occupies a position based on its metabolite composition.

We explored the possibility of detecting metabolite-level changes correlated with AD in children by applying a metabolomic analysis of urine samples. Twenty children with AD, 13 boys and seven girls, aged 6–10 months, diagnosed by a staff physician (D.G.P.) according to the U.K. working party's criteria⁶ and referred to the outpatient clinic of the Allergic Diseases Section of the Department of Life and Reproduction Science, were enrolled in the study. Twelve age-matched children, four male and eight female, without any clinical manifestation of AD or other known acute or chronic disease served as controls. The children were recruited in a narrow age range (6–10 months) to minimize biases due to external factors such as diet variability. It was further expected that the

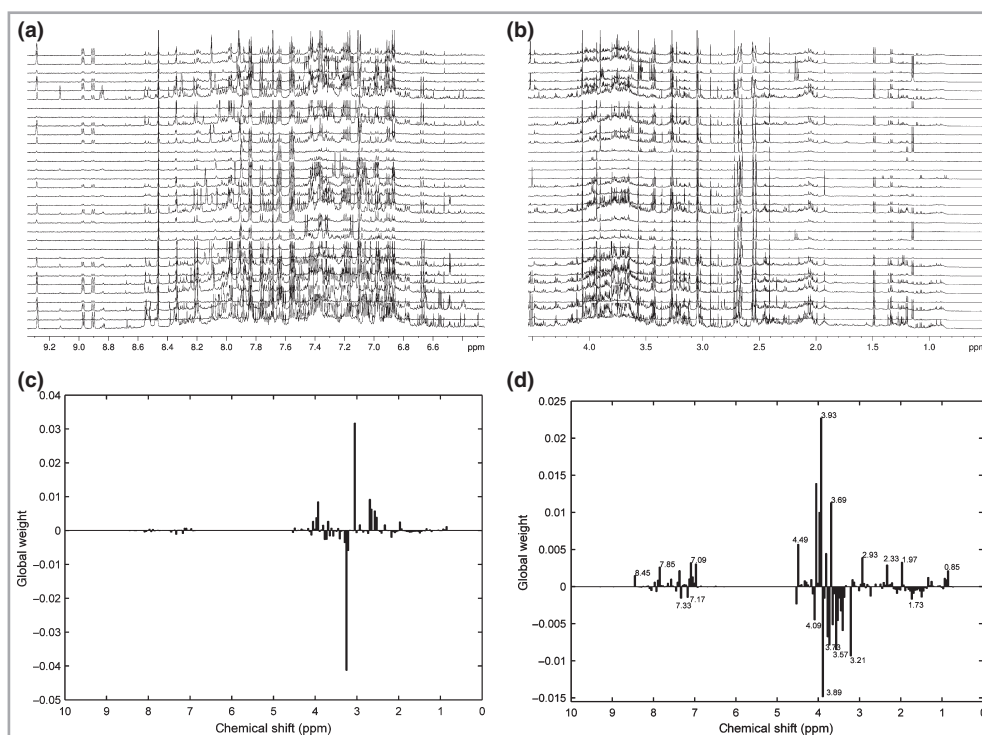


Fig 1. ^1H -nuclear magnetic resonance (NMR) spectra of urine samples showing (a) low-field and (b) high-field regions. In each panel, the lower 20 spectra are measured on atopic dermatitis (AD) samples and the upper 12 spectra are measured on control samples the spectral intensities are not scaled. (c) Standardized global coefficients, or global weights, are reported vs. the original spectral variables (metabolite signals). Positive (negative) values of global coefficients indicate increased levels of the corresponding signals in spectra of the AD (control) group. (d) Same as (c) but refers to a reduced data matrix where the spectral regions (buckets) of strongest signals were removed; significant buckets are labelled by their chemical shift position.

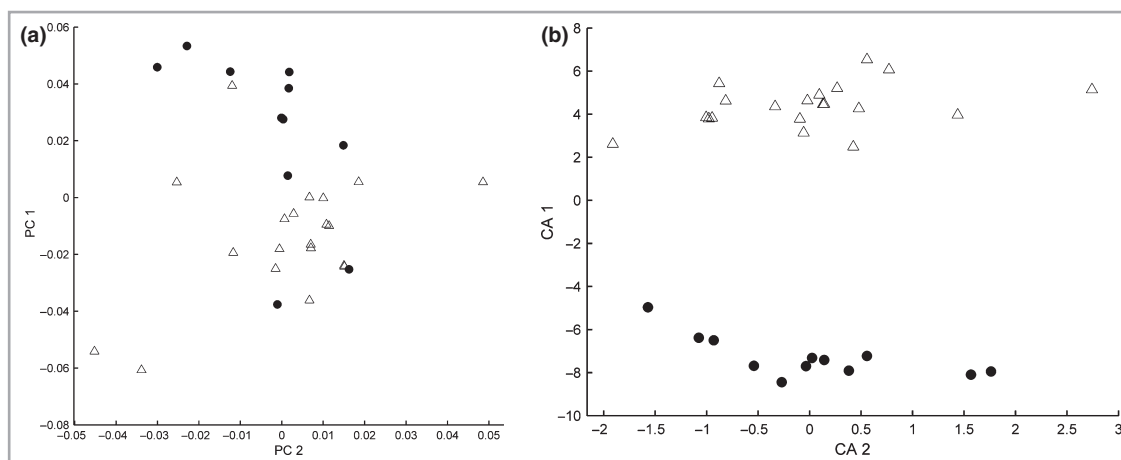


Fig 2. Multivariate statistical analysis. (a) Principal component analysis (PCA): scores plot of the first two principal components (PC) representing 64.1% of the total variance in the data. (b) Canonical analysis (CA): projection of the 178-dimensional ^1H -nuclear magnetic resonance spectral buckets in the PCA/CA subspace. The dimensionality of the dataset is one ($=$ two groups with distinct means), and the second canonical component is displayed only for visual clarity. Empty triangles, samples of the atopic dermatitis group; filled circles, samples of control group.

young age would limit interference from additional confounding factors such as skin infections. Care was taken to avoid selection biases. The parents gave their informed consent. Severity of AD was evaluated by means of the Scoring AD (SCORAD) index.⁷ Urine samples were collected in the early morning and treated according to standardized procedures,⁸ with no specific adjustment made. Additional information about patients and methods is available online as Supporting Information.

All measurements were performed on a Bruker 600 NMR spectrometer (Bruker, Karlsruhe, Germany). The experiments were run in automation, in random sequence, and in duplicate. A single profile was obtained in 10 min. The protocols used conform to the guidelines of the Standard Metabolic Reporting Structures initiative.⁹ The collected 1D ^1H -NMR spectra were segmented in rectangular buckets of fixed width, integrated, normalized to the total area, and mean-centred. The preprocessed data were first subjected to PCA and then to canonical analysis (CA). The latter searches for linear combinations (canonical variables) of the original variables that reflect the largest possible separation between groups. The interpretation of the contribution of the original variables in discriminating groups was based on the calculation of combined standardized loadings from both the PCA and CA transformations. NMR peaks were attributed to metabolites based on reference spectra available in public databases.¹⁰

Each collected spectrum represents the envelope of signals originated by small biomolecules constituting the individual urine metabolome (Fig. 1a,b). In our study population, from the application of an unsupervised pattern recognition approach, i.e. PCA, it was not possible to identify metabolic features associated with AD (Fig. 2a). We also observed that neither age nor sex differences had a particular influence on

the metabolic profiles. Thus, the intergroup variability appears comparable or lower than the interindividual variability. However, by applying a supervised approach, i.e. CA, a striking separation according to health status was obtained ($P < 10^{-5}$) (Fig. 2b).

The standardized global coefficients for the first canonical variable quantify the relative contributions of the original variables to group discrimination (Fig. 1c). In order to assess whether the discriminating power could also be attained on the basis of biomolecules present in lower concentrations, we repeated the procedure using a reduced data matrix where the dominant spectral regions were removed (Fig. 1d). The overall analysis pointed to increased levels of creatinine, creatine, citrate, formate, 2-hydroxybutyrate, dimethylglycine and lactate in AD samples, as well as decreased levels of betaine, glycine and alanine.

It appears that AD produces an unbalance in several pathways of the systemic metabolism, possibly an indirect consequence of the main pathogenic molecular mechanism. However, the still inadequate level of understanding of AD biochemistry renders correlations with metabolic pathways premature, and the genetically heterogeneous nature of the disease may result in different metabolic phenotypes.

In conclusion, this explorative study demonstrates the feasibility of metabolomic investigations in infants based on a noninvasive analysis of urine samples by ^1H -NMR spectroscopy. Our findings substantiate the hypothesis that biofluids from children with AD show altered metabolic contents and open up new perspectives for the characterization of different clinical phenotypes, early differential diagnosis and targeted pharmacological intervention. The heavy impact of AD in children warrants further investigation with methods that can provide more insight than the traditional clinical approaches.

Department of Biotechnology,
University of Verona, Strada Le Grazie 15,
37138 Verona, Italy
*Department of Life and Reproduction Science,
University of Verona, Verona, Italy
E-mail: michael.assfalg@univr.it

M. ASSFALG
E. BORTOLETTI*
M. D'ONOFRIO
R. PIGOZZI*
H. MOLINARI
A.L. BONER*
D.G. PERONI*
G.L. PIACENTINI*

References

- 1 Peroni DG, Piacentini GL, Bodini A et al. Prevalence and risk factors for atopic dermatitis in preschool children. *Br J Dermatol* 2008; **158**:539–43.
- 2 Hanifin JM. Evolving concepts of pathogenesis in atopic dermatitis and other eczemas. *J Invest Dermatol* 2009; **129**:320–2.
- 3 Goodacre R, Vaidyanathan S, Dunn WB et al. Metabolomics by numbers: acquiring and understanding global metabolite data. *Trends Biotechnol* 2004; **22**:245–52.
- 4 Assfalg M, Bertini I, Colangiuli D et al. Evidence of different metabolic phenotypes in humans. *Proc Natl Acad Sci USA* 2008; **105**:1420–4.
- 5 Carraro S, Rezzi S, Reniero F et al. Metabolomics applied to exhaled breath condensate in childhood asthma. *Am J Respir Crit Care Med* 2007; **175**:986–90.
- 6 Williams HC, Burney PG, Pembroke AC et al. The U.K. Working Party's Diagnostic Criteria for Atopic Dermatitis. III. Independent hospital validation. *Br J Dermatol* 1994; **131**:406–16.
- 7 Severity scoring of atopic dermatitis: the SCORAD index. Consensus report of the European Task Force on Atopic Dermatitis. *Dermatology* 1993; **186**:23–31.
- 8 Bernini P, Bertini I, Luchinat C et al. Standard operating procedures for pre-analytical handling of blood and urine for metabolomic studies and biobanks. *J Biomol NMR* 2011; **49**:231–43.
- 9 Lindon JC, Nicholson JK, Holmes E et al. Summary recommendations for standardization and reporting of metabolic analyses. *Nat Biotechnol* 2005; **23**:833–8.
- 10 Wishart DS, Knox C, Guo AC et al. HMDB: a knowledgebase for the human metabolome. *Nucleic Acids Res* 2009; **37**:D603–10.

Funding sources: we acknowledge financial support by the University of Verona and Bruker Biospin Srl (Joint Research Project 2007), and by Fondazione Cariverona (young investigator grant 2007).

Conflicts of interest: none declared.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1. Patient information.

Table S2. Original spectral buckets with significant contribution in group discrimination.

Data S1. Sample collection, sample preparation, nuclear magnetic resonance experiments, data analysis.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by

the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

Liver fibrosis screening for patients with psoriasis taking methotrexate: a cross-sectional study comparing transient elastography and liver biopsy

DOI: 10.1111/j.1365-2133.2011.10657.x

MADAM, Methotrexate remains a commonly used psoriasis treatment and can cause liver fibrosis/cirrhosis. Screening by liver biopsy is established but risks morbidity and mortality, and possible sampling error.¹ Regular monitoring of serum procollagen III N-terminal propeptide (PIIINP) has now reduced the need for liver biopsy,² but PIIINP is a nonspecific marker (it is elevated in normal growing children and in up to 38% of those with inflammatory arthritis).³

Transient elastography (TE) is a noninvasive, ultrasound-based scan measuring liver stiffness, and shows low interoperator variability with minimal training.⁴ TE has been likened to palpating differences in liver stiffness on clinical examination of the liver edge.

A meta-analysis (mainly of hepatitis studies) found that TE was most accurate at discriminating cirrhosis from normal.⁵ Studies combining biomarkers with TE showed improved accuracy.

We assessed whether adding TE to our existing PIIINP monitoring could reduce the need for liver biopsy by detecting fibrosis. The inclusion criteria were psoriasis, ongoing methotrexate (or stopped within 1 month) starting at least 6 months before scan and biopsy, serially elevated PIIINP,² and liver biopsy within 12 months of scanning. The primary outcome measure was the presence or absence of fibrosis at scan and biopsy, which were compared for agreement.

Case selection was by review of hospital databases for histopathology, biochemistry and clinical notes, and referrals from colleagues over 3 years. A consultant radiologist performed the biopsies under ultrasound guidance and specimens were assessed by consultant histopathologists.

Fibroscan® (Echosens, Paris, France) TE machines were used according to the manufacturer's standard operating procedure. A minimum of 10 readings is taken to obtain the median and the whole test takes about 5 min. Sometimes the machines fail to acquire a result, or indicate an unreliable ('invalid') result by showing a large interquartile range (IQR) of scores. Some patients were therefore rescanned later with a newer probe ('XL') designed for improved reliability in obesity.

The scanning cut-off value for normal/fibrotic was set at a conservative 7.1 kPa and a valid/invalid cut-off value for the IQR/median stiffness ratio of < 0.21 (both used in many other studies).⁵ A median liver stiffness of 4.4 kPa has been

Patients and methods

Patients

Table 1. Patients information

ID	Age (months)	Gender	SPT	Total SCORAD
1	10	F	negative	24.50
2	8	M	negative	28.60
3	10	M	negative	27.40
4	8	M	negative	57.50
5	6	M	cow milk	29.10
6	7	M	cow milk, egg	39.50
7	10	M	negative	50.50
8	10	F	negative	16.00
9	9	F	egg	15.80
10	10	M	cow milk, egg	23.70
11	6	M	negative	46.50
12	10	M	cow milk, egg	23.40
13	10	M	cow milk, egg	16.20
14	10	M	negative	44.10
15	6	F	negative	35.00
16	10	M	cow milk, egg	49.00
17	7	F	cow milk, egg	11.50
18	6	F	cow milk	22.70
19	10	M	egg	20.30
20	10	F	negative	22.70

Sample collection

Urine samples were collected between 10:00 a.m. and 12:00 a.m. through the application of a sterile bag. The samples were immediately transferred into a 15 ml tube and centrifuged at 3000 rpm for 20 minutes. Supernatant was collected and stored in 1200 μ l aliquots at -80° C until the following phases of sample preparation.

Sample preparation

Frozen samples were thawed at room temperature and shaken before use. Aliquots of each urine sample (630 μ l) were added to 70 μ l of potassium phosphate buffer (1.5 M K_2HPO_4 in 100% ^2H_2O , pH 7.4), also containing 0.1% sodium 3-(trimethylsilyl)-[2,2,3,3- $^2H_4$]propionate (TSP) and 2 mM sodium azide. Samples were centrifuged at $14,000 \times g$ for 5 min at 4 $^{\circ}C$. 600 μ L of the supernatant were placed in a 5-mm-outer-diameter NMR tube. All chemicals were from Sigma.

NMR experiments

All measurements were performed on a Bruker Avance III NMR spectrometer (Bruker, Germany) operating at 600.13 MHz for ^1H observation, equipped with a TCI cryoprobe. Experiments were run in automation mode after loading individual samples on a Bruker Automatic Sample Changer. A time delay of 5 min was set between sample injection and pre-acquisition calibrations to ensure complete temperature equilibration (300 K). Measurements were repeated once in random order after completion of the first set. For each sample, a one-dimensional NOESY experiment with solvent presaturation was acquired using 64 free induction decays (FIDs), an acquisition time of 2.7 s, a 4 s relaxation delay, and a 10 ms mixing time. The FIDs were multiplied by an exponential weighting function corresponding to a line broadening of 0.3 Hz before Fourier transformation, phasing, and baseline correction. All spectra were referenced to the TSP signal ($\delta = 0.00$ ppm). NMR data were processed using TopSpin 2.1 (Bruker) and visually inspected using Amix 3.8 (Bruker).

Data analysis

^1H -NMR spectra were segmented in rectangular buckets of fixed 0.04 ppm width and integrated, using Amix. The spectral region comprised between 4.55 and 6.25 ppm was discarded because of the variability in the suppression of the water signal and variations in the urea signal. The buckets centered at 1.13 and 1.17 ppm were removed because of the presence of a doublet attributable to propylene glycol, a contaminant present in low concentration. The remaining buckets in the range 0.5-9.5 ppm were normalized to the total area to minimize differences in urine concentration between samples, and subsequently mean-centered.

The pre-processed data were subjected to principal component analysis (PCA) using an available function in Matlab. We applied principal component analysis as an unsupervised pattern recognition and data compression method to gain easy-interpretable information at the descriptive level. The

original data were thus projected into a new multidimensional space where, by definition, the coordinates (principal components) are ranked according to the explained variance in the data. The first 25 principal components described 99.9% of the total variance and were retained. In PCA analysis duplicate samples were always indistinguishable, confirming the reproducibility of the experimental method (not shown). The second stage of the data analysis involved multivariate analysis of variance (MANOVA), as implemented in Matlab. This procedure provides the dimensionality of the group means and the corresponding probabilities. Furthermore, it provides results from a canonical analysis (CA).

Permutation testing was performed using in-house written scripts by assigning random class memberships, repeating the full PCA/CA procedure, and evaluating the probabilities corresponding to the estimated dimensionality of the data. Finally, the bucketed spectra represented in the CA discriminating subspace were back-projected into the original space and comparison between original and reconstructed datasets confirmed that no artificial features were introduced by transformation procedures. The interpretation of the contribution of the original variables in discriminating groups was based on the calculation of combined loadings from both the PCA and CA transformations. The loadings coefficients were standardized by evaluating the pooled within-group covariance matrix.²⁷

Table 2. Original spectral buckets with significant contribution in group discrimination.

	Spectral buckets (center, ppm)
Larger intensities in AD group	8.45, 7.85, 7.09, 6.97, 4.49, 4.05, 3.97, 3.93, 3.81, 3.69, 3.05, 2.93, 2.69, 2.65, 2.57, 2.53, 2.33, 1.97, 1.33, 0.85
Larger intensities in CS group	7.33, 7.17, 4.53, 4.09, 3.89, 3.77, 3.73, 3.65, 3.57, 3.53, 3.41, 3.25, 3.21, 2.73, 1.73, 1.49