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**ABSTRACT**
Autism Spectrum Disorders (ASD) are a group of developmental disorders caused by environmental and genetic factors. Diagnosis is based on behavioral and developmental signs detected before 3 years of age with no reliable biological marker. The purpose of this study was to evaluate the potential use of a 2D NMR-based approach to express the global biochemical signature of autistic individuals compared to normal controls. This technique has greater spectral resolution than to 1D $^{1}$H NMR spectroscopy, which is limited by overlapping signals. The urinary metabolic profiles of 30 autistic and 28 matched healthy children were obtained using a $^{1}$H-$^{13}$C NMR-based approach. The data acquired were processed by multivariate orthogonal partial least-squares discriminant analysis (OPLS-DA). Some discriminating metabolites were identified: β-alanine, glycine, taurine and succinate concentrations were significatively higher, and creatine and 3-methylhistidine concentrations were lower in autistic children than in controls. We also noted differences in several other metabolites that were unidentified but characterized by a cross peak correlation in $^{1}$H-$^{13}$C HSQC. Statistical models of $^{1}$H and $^{1}$H-$^{13}$C analyses were compared and only 2D spectra allowed the characterization of statistically relevant changes [$R^2$Y(cum) = 0.78 and $Q^2$(cum) = 0.60] in the low abundance metabolites. This method has the potential to contribute to the diagnosis of neurodevelopment disorders but needs to be validated on larger cohorts and on other developmental disorders to define its specificity.

***Keywords*** HSQC NMR spectroscopy . Autism spectrum disorders . Urinary metabolites . OPLS-DA . Metabolomics

## 1. Introduction

Autistic disorder (AD), Asperger syndrome (AS) and pervasive developmental disorder-not otherwise specified (PDD-NOS) are collectively termed autism spectrum disorders (ASD). The prevalence of ASD appears to be increasing (1 in 110 children in 2009) [1] without identification of the etiology of this increase [2-5]. Autism is diagnosed in infancy between the second and the third years of life [6]. Autistic children are particularly characterized by a behavioral triad of impaired communication, impaired social interaction, and restricted and repetitive interests and activities, as listed in the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV). Diagnosis is in part made clinically by subjective analyses based on perceived behaviors in the patient, and is thus dependent on the expertise of those administering the tests. Several studies have explored the possibility of using metabolite profiles to contribute to the diagnosis of developmental disorders [7]. As autism disease has many suspected causes including dysfunctions of the gastrointestinal, immunologic and/or neurologic systems, with some markers showing ubiquitous distribution, it could be assumed that urine metabolites profile could help in the diagnosis and in the pathophysiological mechanisms knowledge of the autism pathology [8, 9]. Furthermore urine represents a sample of choice as it is easily and non-invasively collected, that is of particular importance when exploring the autistic population and so we explored in this paper through 2D NMR analysis, the metabolic signature of patients' urine compared to those of healthy subjects in order to identify potential biomarkers useful for the early diagnosis of ASD.

Metabolomics approaches offer the possibility of assessing metabolic signatures linked with genetic and environmental factors. These approaches have previously been applied for disease diagnosis, therapeutics, functional genomics and toxicology studies [10, 11]. The most commonly used analytical platforms to identify and quantify metabolites are gas chromatography combined with mass spectroscopy (GC-MS) and nuclear magnetic resonance spectroscopy (NMR) [12-14]. $^{1}$H NMR spectroscopy is a powerful, rapid analytical method providing a metabolic profile and has already yielded promising results in diagnosing neuropsychiatric disorders such as autism [7]. Unfortunately, a relatively small number of metabolites can be identified by this method, as signals were disturbed by spectral overlap. To improve the ability to identify a mixture of components by NMR, two dimensional NMR (2D-NMR) spectra may be of value. Heteronuclear single quantum coherence ($^{1}$H-$^{13}$C HSQC)-based NMR provides higher resolution than classical 1D-NMR-based approaches [15-18]. The 2D-NMR HSQC results in a 2D map in which the two axes correspond to a $^{1}$H-NMR and a $^{13}$C-NMR spectrum. Furthermore, 2D-NMR reduces the problem of spectral overlap by peak dispersion and results in a higher proportion of resolved peaks, thus increasing metabolite specificity (for example, between 30 to 50 metabolites can be characterized by $^{1}$H NMR [19] compared to more than 150 by HSQC [20] in urine samples). This technique has recently been used in several studies [21-23] to determine plant fingerprinting [17] and animal systems [24], and in human metabolic studies [25, 26] but, to our knowledge, the study reported here is the first 2D HSQC-based NMR study applied to the screening of urine samples of ASD patients and controls.

This report evaluates a 2D-NMR-based approach to explore the metabolic profile of urine in an ASD context. As part of this study, statistical analysis methods [principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA) and orthogonal partial least squares discriminant analysis (OPLS-DA)] were used to reveal metabolites that discriminated between ASD and control populations.

## 2. Materials and Methods

### *2.1. Sample collection*

Between 2008 and 2010, urinary samples were collected in sterile vials untreated with preservative from children aged 6-14 years (mean 8 years) with ASD living in France (n = 30; male 80%, female 20%) and control children (n = 28; male 61%, female 39%) aged 6-9 years (mean 8 years). All study participants provided informed consent. The severity of autism was assessed according to the International Classification of Diseases 10$^{th}$ Edition during medical consultations in three French autism centers [Tours (n = 11), Montpellier (n = 15), and Orléans (n = 4)] and twenty-eight urine samples from healthy volunteers from Tours. Each urine sample was aliquoted in a 1.5 mL Eppendorf tube and stored at -80°C immediately after collection until analysis.

### *2.2. NMR study*

#### *2.2.1. Sample preparation*

Urine samples were thawed out at room temperature, and centrifuged (at 3000 g) for 10 min. Samples were prepared by mixing 500µL of urine supernatant, 100µL of $D_2O$ solution with internal reference [3-trimethylsilylpropionic acid (TSP), 0.05 wt% in $D_2O$] and 100µL of phosphate buffer to obtain pH = 7.4 ± 0.5. Samples then were transferred into conventional 5-mm NMR tubes for $^{1}H$-$^{13}C$ NMR analysis.

#### *2.2.2. NMR spectroscopy experiments*

All NMR experiments were performed at 298 K on a Bruker DPX Avance spectrometer operating at 300 MHz, using a double resonance ($^{1}H$-$^{13}C$) 5-mm (Bruker SADIS, Wissembourg, France).

Standard one dimensional (1D) NMR spectra were acquired using a "zgcppr" pulse program in the Bruker library. 32K data points with 16 scans were acquired using a spectral width of 3001 Hz. Water suppression was achieved during the relaxation delay of 2 s using a waltz 16 decoupling sequence with a decoupling power of 55 dB during the acquisition period. All samples were automatically tuned, matched and shimmed.

All sensitivity-enhanced $^{1}H$-$^{13}C$ HSQC spectra were collected using an "hsqcetgp" pulse program in the Bruker library with 512x128 data points using 32 scans per increment, with acquisition time of 0.0852 s in $t_2$ with a relaxation time of 2.5 s. The spectral widths were set at 20 ppm in the proton dimension and 250 ppm in the carbon. $^{13}C$-decoupling during acquisition was performed using GARP sequence (pulse length 1 ms) applied during $t_2$. The coupling constant $^{1}J_{C\text{-}H}$ was fixed at 145 Hz, using a shine shaped gradient of 80, 20.1, 20.1, and a homospoil gradient pulse recovery delay of 200 µs. Each experiment lasted 3h.

#### *2.2.3. Data processing*

1D and 2D spectra were processed using MestReNova version 7.1.0 software (Mestrelab Research, S.L., Santiago De Compostela, Spain). After Fourier transformation of each FID, 1D spectra were phased manually. All urine spectra were normalized with an external reference [3-

trimethylsilylpropionic acid (TSP), 0.05 wt% in $D_2O$]. TSP served as a chemical shift reference set at 0 ppm and as a quantitative reference signal. Predefined 2D integration regions were established manually from several spectra (all cross-peaks presented in 6 controls and in 6 ASD samples were cumulated). Once this predefined list had been established, HSQC cross-peaks were automatically referenced to the TSP signal, and then were automatically integrated. TSP integration was set at the same value for each spectrum. The region from 4.45 ppm to 5.15 ppm was removed to eliminate baseline effects due to the water signal. The previously established list contained 163 different $^{13}C$ cross-peaks between 10 and 150 ppm.

The signals were assigned, as far as possible, by comparison with the chemical shifts given by the freely available Metabominer software [20] or from literature data [25], with tolerances of 0.05 ppm ($^{1}H$) and 0.1 ppm ($^{13}C$). Identification was achieved if there was only one candidate in the database within the specified tolerances for an observed peak.

All 1D spectra were corrected for phase distortion and the baseline was corrected manually for each spectrum. Two methods of integration were done. The first was an automated "bucketing" with a fixed width of 0.04 ppm per bin. The second method was called "rationalized" integration: 1D proton spectra were prepared as a data matrix by bucket integration from the list obtained from the predefined 2D integration regions. From this predefined 2D auto-integration region, the f2 listing (corresponding to $^{1}H$ dimension) was retained to define the predefined 1D $^{1}H$ f1 auto-integration region. Duplicate buckets having the same $^{1}H$ chemical shift (in f2 listing) were removed. In regions where no $^{1}H$-$^{13}C$ cross-peaks were observed, buckets with constant 0.04 ppm width were defined. As the predefined 2D auto-integration region was established manually taking into account each individual signal, allowing signals with different larger shifts, the 186 resulting buckets were so defined with variable widths (but with a mean of 0.05 ppm).

### *2.3. Statistical Methods*

The intensity of all peaks for all urine samples were studied by multivariate statistical methods, following protocols given in the literature [27, 28].

#### *2.3.1. Multivariate analysis*

Multivariate analysis was performed using Simca-$P^{+}$-12 software (version 12.0, Umetrics, Umeå, Sweden). Unit variance (UV) scaling, pareto scaling (Par), obtained by dividing each variable by the square root of its standard deviation, or logarithmic transformation, which is a nonlinear conversion, were used to minimise the impact of noise or high variability of the variables [29]. Principal component analysis (PCA) [30] was first performed as an unsupervised clustering to identify the similarity or the differences between sample profiles. Grouping, trends and outliers were revealed from the scatter plot. If these outliers were also detected in the distance to model plot (DModX), which was based on residual variance of the model, they were rejected from the model, and a PCA model was rebuilt. In order to identify subsets (linear combinations) of metabolic features associated with a specific sample class (ASD or control), partial least squares (PLS) analysis was used as supervised clustering. PLS derives latent variables which describe the maximum proportion of covariance between measured data (X matrix) and the response variable (Y matrix) [31]. Orthogonal partial least squares discriminant analysis (OPLS-DA), also used for discrimination, is a refinement of this approach: variation in

the data measured is partitioned into 2 blocks, one containing variations that correlate with the class identifier (ASD or control) and the other containing variations that are orthogonal to the first block and thus do not contribute to discrimination between the defined groups [32]. The quality of the models was described by the cumulative modeled variation in the X matrix $R^2$X(cum), the cumulative modeled variation in the Y matrix $R^2$Y(cum), and the cross validated predictive ability $Q^2$(cum) values. Models were rejected if they presented complete overlap of $Q^2$ distributions [$Q^2$(cum) < 0] or low classification rates [$Q^2$(cum)< 0.05 and eigenvalues should be > 2]. The features with variable importance on projection (VIP) values and regression coefficients |CoeffCS[1]| lower than 0.35 were deleted and evaluated again. A number of variables were obtained from PLS-DA and OPLS-DA that were responsible for the difference between ASD and control urine samples for VIP value > 1.0.

One of the main problems with PLS-DA is the data overfitting occurring if the algorithm picks up random noise to real signals. To validate the model, the data are divided into 7 parts, a model is built on 6/7$^{th}$ of data left in, and the left out data are predicted [33]. The predicted data are then compared with the original data and the sum of squared errors calculated for the whole dataset. This is converted into $Q^2$, which is an estimation of the predictive ability of the model. The model was thus considered sufficiently well guarded against overfitting and validated after 200 random permutation tests [34] as the $Q^2$ line intercepted the Y axis at a negative value.

Furthermore, we also performed PLS-DA with autoscaling normalization which is very similar to pareto scaling using the freely available web-based software called MetaboAnalyst [20].

*2.3.2. Univariate analysis*

Student's *t*-test was performed using MetaboAnalyst [20] for all metabolites with a VIP >1.0. The ratio of the peak areas of these metabolites to that of creatinine was calculated and a non-parametric test was performed with the critical *p*-value set at 0.05 [28, 35].

## 3. Results

*3.1. NMR Experiments*

A typical 2D HSQC spectrum of urine fitted with the $^1$H-NMR sub-spectrum on its side is shown in Figure 1. The 2D-HSQC analysis provided 163 cross-peaks integrated and normalized with an external reference (TSP).

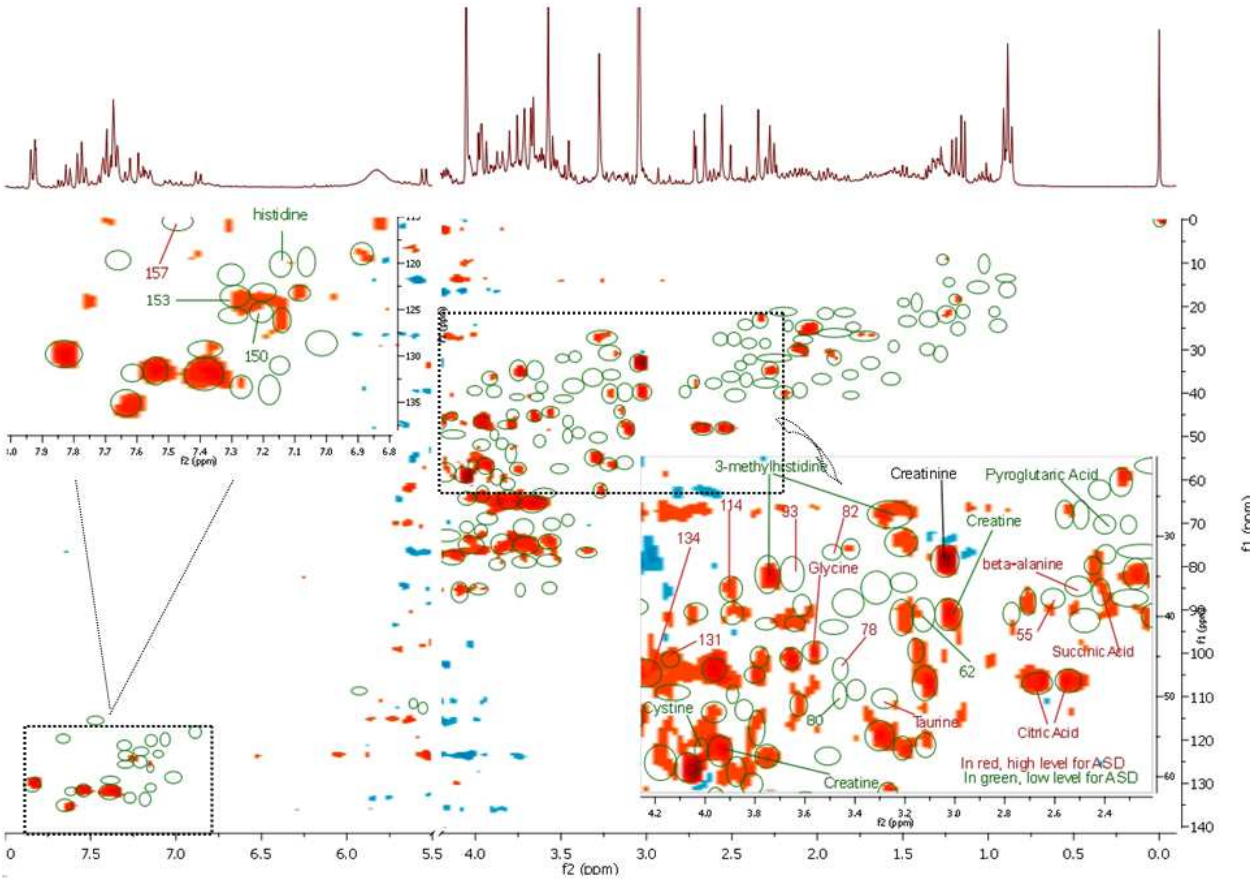


**Fig. 1.** $^{1}$H-$^{13}$C HSQC NMR spectrum of urine at 300 MHz, with a typical $^{1}$H spectrum as external spectrum, showing the assignment of the significant metabolites responsible for discriminating children with ASD from non-autistic children when possible. Only VIP >1 obtained by OPLS-DA (model 4) were distinguished. Peaks increased in the ASD group are in red, and those decreased are in green.

*3.2. Statistical studies from 2D HSQC NMR data – choice of pre-treatment technique*

PCA was performed as unsupervised clustering and we did not identify any particular similarity or any great differences between sample profiles (no outliers were eliminated). Discriminating metabolites were proposed using PLS-DA and OPLS-DA from one predictive and two or more orthogonal components. We have studied the impact of pre-treatment of the NMR data before multivariate analysis using unit variance (UV), pareto (Par), and autoscaling. In parallel, the impact of log-based transformation was performed.

**Table 1**
Summary of statistical values of PLS-DA and OPLS-DA of $^{1}$H-$^{13}$C HSQC with different data scaling. The different cumulated modeled variations in X [$R^2$X(cum)) and Y ($R^2$Y(cum)] matrices on spectral datasets and predictability of the model ($Q^2$) are given [observations (N)=58].

| Scaling/transformation | $R^2$X | $R^2$X (cum) | $R^2$Y (cum) | $Q^2$ (cum) |
|---|---|---|---|---|

| | Scaling/transformation | $R^2X$ | $R^2X$ (cum) | $R^2Y$ (cum) | $Q^2$ (cum) |
|---|---|---|---|---|---|
| Model 1 [a] | UV | 0.0533 | 0.290 | 0.784 | 0.399 |
| Model 2 [b]: | UV | 0.0488 | 0.271 | 0.782 | 0.602 |
| Model 3 [c] | Pareto | 0.0593 | 0.598 | 0.700 | 0.428 |
| Model 4 [d] | log transformed, UV scaling | 0.0525 | 0.155 | 0.758 | 0.544 |

[a]: PLS-DA, from 3 components, Variables X=63
[b] OPLS-DA, Variables X=43, 2 orthogonal projections
[c]: OPLS-DA, Variables X=54, 3 orthogonal projections
[d]: OPLS-DA, , Variables X=44, 1 orthogonal projection

Table 1 summarizes the features of the different models. The processed variables are represented by X (metabolites), Y variables represent the different children's urine samples. $R^2X$(cum) is the sum of predictive orthogonal variations in X that is explained by the model. From the predictive variation between X and Y given by $R^2X$(cum), models 1 and 2 with the same scaling (UV) interpreted around 30% of the total variation in X (0.29 and 0.27, respectively, Table 1). The amount variation that could not be explained by the model might originate from the noise. This variation was minimized by Pareto scaling and for model 3 is expressed by the formula [36]: 1 - $R^2X$ - $R^2X$(cum) = 1 - 0.0593 - 0.598 = 0.343, as noise could account for 34% in this analysis. The quality of the models was expressed by $R^2Y$(cum) and $Q^2$(cum) values, where $R^2Y$(cum) is defined as the proportion of variance in the data explained by the models and indicates goodness of fit. All the models explained around 70% to 80% of the variations in the different peaks. A high $Q^2$(cum) value [$Q^2$(cum) > 0.5] indicated good predictivity. Table 1 shows that noise was minimized by Pareto scaling [$R^2X$(cum) higher in models 3], but this pre-treatment led to lower predictability [$Q^2$(cum) = 0.43]. As UV scaling seemed to be the best scaling in our study (confirmed by analysis of variance CV-ANOVA, *p*-value = 7.773.e-009, see supporting information), we focused on this method. Thus the OPLS-DA cross-validated score plots for model 2 (Fig. 2) showed discrimination between the two populations.

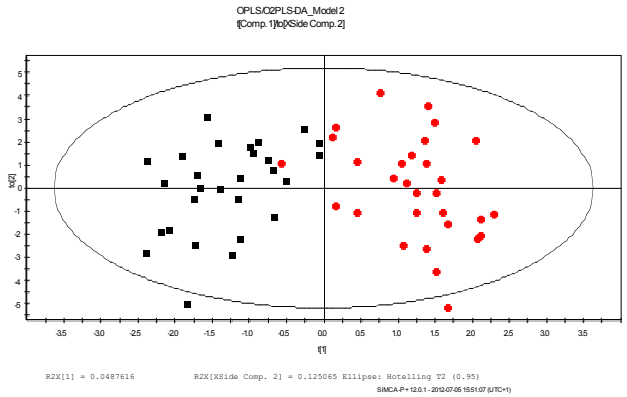


**Fig. 2.** Scatter plot of OPLS-DA scores of the first principal component obtained from $^1H$-$^{13}C$ HSQC NMR spectra of urine samples, model 2 (UV scaling, Table 1) [(n=30 ASD samples (red dot), n=28 control samples (black box) with $R^2Y$(cum) = 0.78 and $Q^2$(cum) = 0.60].

*3.3. Statistical studies from 2D HSQC NMR data – Analysis of model and identification of important features*

From PLS-DA or OPLS-DA, the most highly contributing metabolites in the discrimination of the 2 populations were screened recording to the variable importance on projection (VIP) values >1.0. The effect of a data pre-treatment method on the ranking of the metabolites is presented in Table 2. For example, a signal characterized by peak ID 123 was identified as the most relevant for PLS-DA and OPLS-DA methods using UV scaling, or log transformed, but was the 6$^{th}$ rank with Pareto scaling (Table 2, model 3). As another example, pyroglutamic acid (peak ID 46) was the 6$^{th}$ highest metabolite on OPLS-DA UV scaling, while by log transformation, pyroglutamic acid appeared after the first 15 most relevant metabolites when applying rank scaling. The differences could be due to the magnitude of the fold change (or relative standard deviation) of some metabolites present that was balanced by nonlinear log transformation. Furthermore, a PLS model, with autoscaling normalization, was also tested and provided the same VIP as shown in Table 2 (model 5).

The loading scatter plot in Figure 3 shows which variables led to discrimination between ASD and control children in model 2 (Table 1), the chosen model.

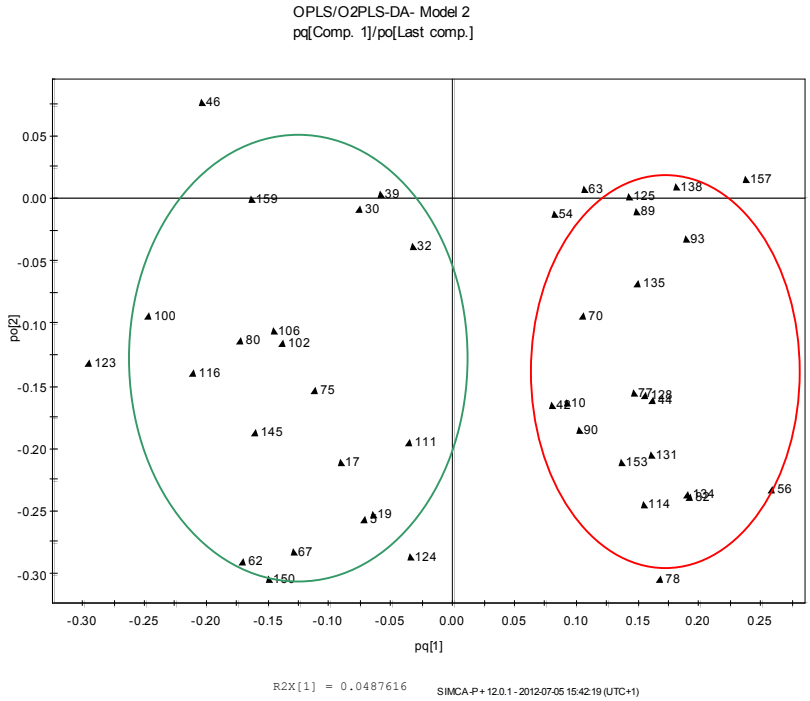


**Fig. 3.** pq loading plot of OPLS-DA model 2 (Table 1): scatter Plot of the X- and Y-loadings (p and q). This plot shows how the responses (Y's) varied in relation to each other, i.e. which provided similar information and their relationship to the terms of the model. Two tendencies could be seen. Peaks framed in red were higher levels in ASD urine, and peaks framed in green were lower.