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Identification of metabolic pathway disturbances using multimodal metabolomics in autistic disorders in a Middle Eastern population

Tania Bitar[a,b], Sylvie Mavel[a,*], Patrick Emond[a], Lydie Nadal-Desbarats[a], Antoine Lefèvre[a], Hanna Mattar[c], Michel Soufia[c], Hélène Blasco[a,d], Patrick Vourc'h[a,d], Walid Hleihel[b], Christian R. Andres[a,d]

[a] Université François-Rabelais, INSERM U930, Equipe neurogénétique et neurométabolomique, France
[b] Faculty of Science-Holy Spirit University of Kaslik (USEK), Lebanon
[c] Faculty of Medicine and Medical Sciences-Holy Spirit University of Kaslik (USEK), Lebanon
[d] Laboratoire de Biochimie et Biologie moléculaire, CHRU de Tours, France

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ABSTRACT

We analyzed for the first time the metabolic profile of Lebanese children affected by autistic disorders to compare this profile to other metabolomics studies and to identify the associated metabolic disturbances. Urine samples of 40 patients with Autism spectrum disorder (ASD) and 40 healthy matched controls were analyzed using nuclear magnetic resonance (NMR) and liquid chromatography coupled to high-resolution mass spectrometry (LC–MS). Multivariate analysis on analytical data fusion was conducted on the training set of 50 urine samples, and then validated with a test set of 30 samples, this repeated 10 times. The model was also evaluated using a receiver operating characteristic curve showing a specificity and a sensitivity of 86% and 80%, respectively. Among the most significant metabolites that contributed to the discrimination between ASD and controls, we confirmed the perturbations of tyrosine, 2-hydroxybutyrate, creatine and glutamate. We found new metabolites such as trigonelline, cysteic acid and guanine. We found metabolic perturbations including amino acids, carbohydrates and oxidative stress pathways which added value for the contribution of known metabolic disturbances in ASD observed in populations of other ethnic and geographic origins.

1. Introduction

According to the most recent version of the Diagnostic and Statistical Manual of Mental Disorders DSM-5, autism spectrum disorders (ASD) are a group of neurodevelopmental disorders, characterized by impaired communication and social interaction as well as restricted, repetitive behaviors and activities [1]. The prevalence of ASD is estimated to affect about 1 per 160 children, according to the World Health Organization [2] and 1 per 66 children in Lebanon [3].

To date, the etiology of ASD remains largely unclear despite a huge effort mainly in genetics and neuroimaging. ASD is probably a multifactorial disorder with genetic and environmental factors [4]. Several studies have shown metabolic disturbances in individuals with ASD compared to the general population [5,6]. Imbalance in the level of certain amino acids in the plasma, platelets, urine or cerebrospinal fluid has been identified in autistic subjects [7]. Some of the most common metabolic abnormalities in ASD are those of redox and mitochondrial metabolism [8,9]. Furthermore, many neurometabolic disorders such as phenylketonuria, creatine deficiency, altered metabolism of purine, disorders in the metabolism of neurotransmitters and hormones including serotonin, catecholamines, melatonin and GABA [10] have been associated with autism. Metabolomics approach provides metabolic patterns useful to identify biomarkers. There are two approaches in metabolomics: the targeted analysis consisting of the measurement of defined

Abbreviations: ASD, autism spectrum disorders; CV, coefficient of variation; CV-ANOVA, cross-validation analysis of variance; ESI, electrospray ionization; GC–MS, gas chromatography coupled to mass spectrometry; ^{1}H NMR and ^{1}H-^{13}C NMR, nuclear magnetic resonance of proton and proton-carbon; LC-HRMS, liquid chromatography coupled to high resolution mass spectrometry; NGOs, non-governmental organizations; OPLS-DA, orthogonal partial least squares discriminant analysis; PCA, principal component analysis; PNP, purine nucleoside phosphorylase; QC, quality control; ROC, receiver operating characteristic; TSP, trimethylsylilpropionic acid; UV, unit variance; VIP, variable importance in projection.

* Corresponding author at: Université François Rabelais, INSERM U930, 31 Av. Monge, 37200, TOURS, France.
E-mail addresses: tania.bitar@etu.univ-tours.fr (T. Bitar), sylvie.mavel@univ-tours.fr (S. Mavel), emond@univ-tours.fr (P. Emond), lydie.nadal@univ-tours.fr (L. Nadal-Desbarats), antoine.lefevre@univ-tours.fr (A. Lefèvre), hannamattar@usek.edu.lb (H. Mattar), michelsoufia@usek.edu.lb (M. Soufia), helene.blasco@univ-tours.fr (H. Blasco), patrick.vourch@univ-tours.fr (P. Vourc'h), walidhleihel@usek.edu.lb (W. Hleihel), christian.andres@univ-tours.fr (C.R. Andres).

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groups of chemically characterized and biochemically annotated metabolites, and the untargeted analysis which is the unbiased comprehensive analysis of all the metabolites in a sample [11]. Due to the abundance and the chemical diversity of the metabolites, no single analytical platform can cover the complete range of human metabolome. Analytical platforms frequently used in order to identify and quantify metabolites, are mass spectrometry coupled to separation techniques like gas chromatography (GC–MS) or liquid chromatography (LC–MS) [12] and nuclear magnetic resonance (NMR). Previous researches have suggested that the study of urine metabolites profiles could be an effective tool for diagnosis and for understanding the physiopathology of autism [13–15]. However, most of the studies have focused on Western autistic populations, and as far as we know, none of it has ever taken place on a Lebanese Middle-East population.

The aim of our work is to evaluate the metabolic profile of urine in an ASD Lebanese population by using the complementarity of analytical platforms, NMR 1D (^{1}H), NMR 2D (^{1}H-^{13}C) and LC–MS, in a targeted approach to compare their specific metabolic profiles to previously studied populations, and to identify the metabolic pathways associated with the disorder, for a better understanding of this pathology.

2. Material and methods

2.1. Sample collection

Autistic patients were diagnosed and enrolled in the study using Diagnostic and Statistical Manual of Mental Disorders, 4th edition [16] criteria and Childhood Autism Rating Scale (CARS). The average score on the CARS for the patient's sample was in favor of moderate autism. The majority of the participants presented with a moderate intellectual disability as reported in their records following IQ measures using standardized and validated tools (WISC, WPPSI). Patients were selected through specialized institutions and non-governmental organizations (NGOs) specialized in mental disorders in all the districts of Lebanon. Controls were selected from schools located in all the districts of Lebanon. All families and participants provided informed consent. First-morning urine samples were collected from 40 autistic children (30 males and 10 females) and 40 controls (29 males and 11 females) matched for age, gender and geographic location (Beirut, North, South, Mount Lebanon and Beqaa). All urine samples were centrifuged at 2000*g* for 5 min, the supernatant distributed in volumes of 5 mL and stored at −80 °C. An aliquot of each sample was done for creatinine analysis done by the Jaffe method (Olympus AU640, France) to evaluate the dilution effect.

2.2. NMR study

2.2.1. Sample preparation

Two mL of urine samples were first lyophilized (FreeZone ® 4.5 L, Labconco, USA) at −107 °C, 0.2 mbar. Then, 300 μL of phosphate buffered deuterium oxide (D2O) (pH = 7.4 ± 0.5) and 8 μL of an external reference [trimethylsylilpropionic acid (TSP), 0.05 wt% in D2O] were added. After vortexing and centrifugation for 3 min at 1500*g*, the supernatant was transferred into 3 mm NMR tubes for NMR analysis.

2.2.2. NMR spectroscopy analysis

NMR analysis was performed using a Bruker Advance III HD (BrukerSadis, Wissembourg, France) operating at 600 MHz. Spectra were acquired using a "noesypr 1d" pulse program with a relaxation delay of 20 s for 1D analysis, and a "Hsqcgpphpr" pulse sequence for 2D (^{1}H-^{13}C) NMR. The region containing the water signal was removed from each spectrum.

2.2.3. Data processing for targeted NMR

^{1}H NMR spectra were processed as previously described [17] using TopSpin version 3.2 software (Bruker Daltonik, Karlsruhe, Germany). Spectra were integrated within a range of 0–10 ppm using AMIX software (Analysis of Mixture, version 3.9.14, Bruker, Karlsruhe, Germany), excluding the water region (4.67–5.00 ppm), with buckets of various width from 0.01 to 0.15 ppm. These buckets (28 buckets) corresponded to single metabolites. The signal intensity in each bucket was normalized to the total sum of peak intensities. Identification of metabolites was achieved using Chenomx software (Chenomx Inc, Edmonton, Canada) and in-house database.

For NMR 2D (^{1}H-^{13}C), calibration and integration were processed as previously described [17] using MestRenova Software (Mestrelab research, Santiago de Compostela, Spain). The external reference TSP served as a reference set at 0 ppm. Each region was integrated manually from several spectra (all cross-peaks presented in 15 controls and in 15 ASD spectra were cumulated). The final 2D matrix contained 542 cross-peaks, then normalized by the total sum of cross-peak intensities and served for statistical analysis. These cross-peaks were then identified using MetaboMiner database [18] and in-house database.

2.3. LC-HRMS study

2.3.1. Sample preparation

Twenty μL of urine were diluted in water (160 μL) and 20 μL of internal standard (Imipramine) were also added for all urine samples. After vortexing and centrifugation at 10 000*g* for 10 min, 150 μL of the supernatant were transferred into a 96-well plate. In order to equilibrate the chromatographic system, twenty quality controls (QCs) [obtained by mixing an equal volume of all urine samples (20 μL)] were injected. The sequence of samples for analysis was randomized, QCs were analyzed every 10 samples and at the end of the run. Data quality was checked by principal component analysis PCA (see Supporting Information, Fig. A1).

2.3.2. LC-HRMS analysis

LC-HRMS analysis was performed as previously described [17], using a UPLC Ultimate 3000 system (Dionex), coupled to a Q-Exactive mass spectrometer operated in positive and negative electrospray ionization (ESI) mode. The system was controlled by X-calibur 2.2 (Thermo Fisher Scientific). UPLC separations were achieved using a Phenomenex Kinetex 1.7 μm XB-C18 (150 mm × 2.10 mm) column. Mobile phase A consisted of 0.1% formic acid in water and mobile phase B consisted of 0.1% formic acid in methanol. The gradient [17] operated at a flow rate of 0.4 mL/min over a run time of 30 min. During the full-scan acquisition, which ranged from 60 to 900 *m*/*z*, the instrument operated at 70 000 resolution (*m*/*z* = 200).

2.3.3. Data processing for targeted LC-HRMS

A library of standard compounds (Mass Spectroscopy Metabolite Library of standards MS ML®, IROA technologies) was analyzed with the same gradient of mobile phases and in the same conditions as those used to analyze urine samples. The annotation of selected features was validated from retention time, and high resolution mass molecular (*m*/*z*), using several criteria, as previously described [19]. Targeted molecules (367 molecules detected in ESI+, 255 in ESI-) were selected and integrated into Xcalibur 2.2 (Thermo Fisher Scientific, San Jose, CA). Each peak area was normalized to the total peaks area of each chromatogram (MSTU post-acquisition normalization). Coefficients of variation (CV) for