

Title: Urinary metabolic phenotyping differentiates children with autism from their unaffected siblings and age-matched controls

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Abstract

Autism is an early-onset developmental disorder with a severe life-long impact on behavior and social functioning that has associated metabolic abnormalities. The urinary metabolic phenotypes of individuals (age range=3-9 years old) diagnosed with autism using the DSM-IV-TR criteria (n=39; male=35; female=4), together with their non-autistic siblings (n=28; male=14; female=14) and age-matched healthy volunteers (n=34, male=17; female=17) have been characterized for the first time using ^1H NMR spectroscopy and pattern recognition methods. Novel findings associated with alterations in nicotinic acid metabolism within autistic individuals showing increased urinary excretion of *N*-methyl-2-pyridone-5-carboxamide, *N*-methyl nicotinic acid and *N*-methyl nicotinamide, indicate a perturbation in tryptophan-nicotinic acid metabolic pathway. Multivariate statistical analysis indicated urinary patterns of the free amino acids glutamate and taurine were significantly different between groups with the autistic children showing higher levels of urinary taurine and a lower level of urinary glutamate indicating perturbation in sulfur and amino acid metabolism in these children. Additionally, metabolic phenotype (metabotype) differences were observed between autistic and control children, which were associated with perturbations in the relative patterns of urinary mammalian-microbial co-metabolites including dimethylamine, hippurate and phenylacetylglutamine. These biochemical changes are consistent with the known abnormalities of gut microbiota found in autistic individuals and the associated gastrointestinal dysfunction and may be of value in monitoring the success of therapeutic interventions.

Introduction

Autism spectrum disorders (ASD) represent a series of related highly complex socio-psychological and neurodevelopmental problems with associated metabolic and gastrointestinal abnormalities of poorly-defined etiology. ASD typically develop during the first 3 years of life and are characterized by a myriad of deficits in language/communication skills, social detachment as well as repetitive and stereotypic behaviors.^{1, 2} The etiopathology of ASD is multifactorial and has been linked to genetic abnormalities^{3, 4} and inborn errors of metabolism but there are many postulated, largely ill-defined, triggers including infectious agents and environmental toxins.⁵ Autism has been shown to have strong associations with various metabolic abnormalities, immunological function and gastrointestinal disturbances, although their mechanistic significance is unknown.⁵⁻⁸

In addition to the panel of neurodevelopmental problems associated with ASD, a range of gastrointestinal disorders have been reported and recent studies have found that the condition is associated with abnormal gut microbiota.⁹ There is also the possibility of previously unrecognized etiologic connections between microbiome disorder and childhood developmental problems, given the importance of the microbiome in mammalian metabolism e.g. bile acid metabolism.¹⁰ Individuals with ASD are commonly exposed to repeated courses of multiple antibiotic therapies and this may contribute to the complex relationships between gastrointestinal dysbiosis and ASD by altering the composition or stability of their microbiota.¹¹⁻¹³ Abnormal sulfur metabolism has also been shown to typify individuals with ASD.¹⁴ Waring et al. showed that individuals with autism have lower levels of plasma sulfate, but considerably elevated levels of urinary sulfate, as compared to non autistic individuals. These data suggest that autistic individuals may have impaired detoxification potential involving sulfation, as evidenced by their inability to sulfate the widely used drug acetaminophen.¹⁴

The prevalence of autism has increased from 4 in 10 000 children before 1980^{2, 15} to 99 in 10000 in 2009 in the United Kingdom¹⁵ and 53 in 10 000 in

2006 in the United States¹⁶ alone, but this varies regionally and with ethnicity, and also some geographically localized areas have much higher incidences of ASD.¹⁷ However, it is not clear whether the global increase is due to higher prevalence of the disorder, and/or improved early detection/diagnosis. Current diagnosis of ASD is subjective and depends on observations of a cluster of behaviors and fulfillment of multiple criteria set out in the Diagnostic and Statistical Manual of Mental Disorders 4th edition (DSM-IV-TR)¹⁸ by a trained clinician. At present, there are no reliable biochemical- or genetic-screening tests for the disorder and in some cases, particularly in late onset autism, childhood development can switch from being normal to showing a delay in acquisition of new skills, thus adding to the difficulty for diagnosing ASD. Thus, there is a pressing need for new diagnostic tools for ASD that are both sensitive and reliable, since early diagnosis can lead to timely interventions and optimized clinical management.

Metabonomic approaches offer the possibility of measuring metabolic end points (metabolic profiles) that are determined by genetic and environmental factors.^{19, 20} The application of high throughput metabolic profiling methods using high resolution analytical platforms (nuclear magnetic resonance (NMR) spectroscopy and/or mass spectrometry (MS)) with subsequent multivariate statistical analyses now provides a well-established strategy for differential metabolic pathway profiling and disease diagnosis.^{10, 20-22} Here we apply a metabolic profiling approach to capture the global biochemical signature of autistic individuals using NMR spectroscopy with multivariate statistical modeling to characterize individuals with autism, unaffected siblings and unrelated control healthy comparison groups in population-based samples. The key differential metabolic features of autism derived from the current study can subsequently be evaluated as candidate diagnostic markers in further studies.

Methods

Sample Collection. The urine samples used in this study were obtained from University of South Australia (autistic subjects, their siblings and unrelated

healthy subjects; age range: 3-9 years old) and a second group of controls from the Swiss Tropical Institute (comparable healthy subjects; age range: 3-9 years old), and complied with Australian and Swiss local and national regulations on ethics. Each individual and their family gave informed consent for the study to take place. The autistic subject group comprised only individuals who had been diagnosed as being autistic according to the DSM-IV-TR criteria for diagnosis of autism disorder or Asperger syndrome.¹⁸

To address bias deriving from geographical location, urine samples from the second group of controls were profiled together with the first group of controls. The two control data sets were shown to be statistically indistinguishable (data not shown).

Sample Preparation. Urine samples were prepared by mixing 400 μ l of urine with 220 μ l of a phosphate buffer (90% D₂O, 1 mM 3-trimethylsilyl-1-[2,2,3,3-²H₄] propionate (TSP), and 3 mM sodium azide; pH 7.4) and left to stand for 10 min. The samples were centrifuged at 11000 g for 10 min and 600 μ l of the supernatant for each sample was then transferred into 5 mm (outer diameter) NMR tubes.

¹H NMR Spectroscopy. Spectra were obtained on a Bruker Avance600 spectrometer (Bruker Biospin; Rheinstetten, Germany) at 600.13 MHz (ambient probe temperature 27°C). A standard 1-dimensional (1D) pulse sequence was used [recycle delay (RD)-90°- t_1 -90°- t_m -90°-acquire free induction decay (FID)]. The water signal was suppressed by irradiation during the RD of 2 s, the mixing time (t_m) of 150 ms. t_1 was set to 3 μ s and the 90° pulse length was ~10 μ s. For each sample, a total of 128 transients were accumulated into 32k data points using a spectral width of 20 ppm. Prior to Fourier transformation, all FIDs were multiplied by an exponential function equivalent to a line broadening of 0.3 Hz. The assignment of the peaks to specific metabolites was based on 2-dimensional (2D) ¹H-¹H correlation spectroscopy (COSY) and ¹H-¹H total correlation spectroscopy (TOCSY) NMR spectra, published literature,^{23, 24} and statistical total correlation spectroscopy (STOCSY).²⁵

Data Processing and Analysis. ^1H NMR spectra of urine samples were manually phased and baseline corrected using XwinNMR 3.5 (Bruker Biospin; Rheinstetten, Germany). The ^1H NMR spectra were referenced to the TSP resonance at δ 0.0. Spectra were digitized using a MATLAB (version 7, The Mathworks Inc.; Natwick, MA, USA) script developed in-house. The regions containing the water and urea resonances (δ 4.5 – 6.5) were removed from each spectrum to eliminate baseline effects of imperfect water saturation. For each spectrum, a recursive peak alignment algorithm²⁶ was applied to minimize spectral peak shifts due to residual pH differences within samples prior to normalization to the total area sum of the residual spectrum and subsequent pattern recognition analyses.

Principal components analysis (PCA) was applied to the unit variance (UV)-scaled spectral data to reveal intrinsic autism-related patterns. Projection to latent structure discriminant analysis (PLS-DA) with UV-scaled spectral data was also carried out in order to improve classification of the different groups of individuals as well as to optimize the identification of changes in urinary metabolites that were unique to a particular group. Permutation testing (200 permutations of the class parameters) was performed on the PLS-DA models to ensure statistical model validity. Inter-individual variation can confound data interpretation, particularly in multivariate data of high dimensionality. Therefore, orthogonal projection to latent structure discriminant analysis (O-PLS-DA)²⁷ was also performed on UV-scaled spectral data to optimally model class differences and to systematically identify metabolites contributing to the differences between autistic, siblings and control groups. This is achieved by removing systematic variation in the profiles that does not relate to autism, thereby enhancing the interpretability of the models. Back scaling transformation of the O-PLS coefficients to the covariance matrix was also used to increase the interpretability of the model.²⁸ Here, the colors projected onto the coefficient plot indicate the correlation of the intensities of the metabolites discriminating each class from the corresponding controls. Red indicates a high correlation and blue denotes no correlation with sample class. The direction and magnitude of the signals relate

to the covariation of the metabolites with the classes in the model and the model predictive performance (robustness) was evaluated using a 7-fold cross validation method as defined by the Q^2 (goodness of prediction) parameter.²⁸ In addition, the statistical significance and validity of the O-PLS-DA results were calculated using a permutation test (number of permutations = 10000).^{29, 30} The significance of each metabolite from the permutation test is interpreted using the covariance plot, where the colors projected onto the spectrum indicate the significance of the metabolites with blue indicating no significance difference at $p > 0.05$ confidence levels and red indicating high significance difference at $p < 0.0001$; the direction and magnitude of the signals relate to the covariation of the metabolites with the classes in the model.

Calculation of concentration ratios of selected urinary metabolites to creatinine. The relative mean concentrations of a range of urinary metabolites such as 4-cresol sulfate (δ 2.35), creatine (δ 3.93), glycine (δ 3.56), hippurate (δ 7.84), *N*-methyl nicotinic acid (NMNA) (δ 4.45), *N*-methyl nicotinamide (NMND) (δ 4.48), phenylacetylglutamine (PAG) (δ 7.43), succinate (δ 2.41) and taurine (δ 3.43) were calculated from the peak area of the designated NMR signals for the metabolites listed above using a MATLAB script developed in-house to partially overcome any concentration effects caused by different urine volumes. The ratios of the peak areas of these selected metabolites to that of creatinine (δ 4.04) were then calculated and a non-parametric 2-tailed Mann-Whitney test was performed to calculate the significance level of class differences based on the ratios between groups.

Results

Urinary ^1H NMR spectroscopic profiles of the cohort. Urinary 600 MHz ^1H NMR median spectra of individuals from the 3 groups (Figure 1) contained peaks from a wide range of low-molecular-weight metabolites of diverse chemical classes (typically <1 kDa) from both mammalian and associated gut-microbial metabolism. The urinary NMR spectra were dominated by dietary and microbial-derived methylamines (dimethylamine (DMA), trimethylamine *N*-oxide (TMAO))

and aromatic substances such as hippurate, phenylacetylglutamine (PAG) and 4-cresol sulfate (Figure 1). Additionally, mammalian metabolites such as citrate, succinate, creatinine, lactate, α -hydroxyisobutyrate and amino acids (particularly alanine, glutamine and glycine) were also apparent. Visually, allowing for the inter-individual variability, the urinary spectra were very similar, but the autistic individuals showed subtle differences in urinary succinate, *N*-methyl nicotinic acid (NMNA) and *N*-methyl nicotinamide (NMND) compared to the controls, as evidenced from the median spectra shown in Figure 1.

Multivariate statistical analysis of the NMR spectral data. To further explore the metabolic differences between the three groups of participants, multivariate statistical analyses were employed on the full resolution NMR data set consisting of 34 controls, 28 siblings and 39 autistic urine samples to extract useful metabolic information. PCA was carried out on UV-scaled data to identify any inherent differences within the dataset. The resulting scores plot of PC1 versus PC2 (Figure 2A) showed no clear differences between the three groups, nor did examination of all pairs of lower PCs up to 3 PCs provide any discrimination indicating that the major source of variation in the data was not related to autism, but was rather dominated by inter-person variability. However, by utilizing group information in PLS-DA analysis, systematic differences could be observed between the three groups (Q^2 : 15%; R^2 (goodness of fit): 65.7%). The corresponding cross-validated PLS-DA scores plot (Figure 2B) showed clear separation between autistic individuals and the controls and partial separation between siblings and the controls. PLS-DA pair-wise comparison between controls and siblings (Figure 2C; Q^2 : 31.4%, R^2 : 86%), and between controls and autistics (Figure 2D; Q^2 : 33.5%, R^2 : 72%) suggested a robust metabolic difference between the classes in each pair-wise comparison (Figure 2C and D respectively) on the first component.

To minimize any effects of non-relevant metabolite variability, pair-wise O-PLS-DA analyses were carried out on the UV-scaled NMR data set to identify urinary metabolites contributing to the differences between groups. Systematic comparisons of the autistics and normal control groups using O-PLS-DA

generated a model with a Q^2 of 15% and R^2 of 27.5%. The corresponding coefficients plot (Figure 3) indicated differences in the urinary metabolic profile between the two groups with the autistics showing relatively higher levels of urinary acetate, DMA, *N*-acetyl glycoprotein fragments (NAG), succinate, taurine, NMNA, NMND and *N*-methyl-2-pyridone-5-carboxamide (2PY), whilst the healthy controls showing higher levels of urinary glutamate, hippurate, PAG, and 4-cresol sulfate. Comparison between siblings and the controls, and between siblings and autistics, using O-PLS-DA, showed no differences in the urinary metabolic profile (data not shown) and the models generated were very weak (negative Q^2). Permutation tests were carried out on the pair-wise area-normalized NMR data to statistically validate the results obtained from the O-PLS-DA analyses. Pair-wise comparison between the controls and autistic individuals showed statistical significance at a $p < 0.0001$ significance level (Figure 4) on all metabolites identified to be contributing to the differences between controls and autistics in the O-PLS-DA analyses (Figure 3). However, permutation tests carried out on the controls and siblings failed to find statistical significance in the urinary metabolites (data not shown), which is reflective of the weak Q^2 and R^2 values generated from the O-PLS-DA model.

Ratios of selected urinary metabolite concentrations against creatinine.

The areas of selected NMR peaks from a range of urinary metabolites determined to be significantly different between groups *via* multivariate statistical modeling, such as creatine, creatinine, glycine, hippurate, NMNA, NMND, PAG, 4-cresol sulfate, succinate and taurine were quantitated and ratios obtained relative to that of the methylene peak of creatinine. The significance of the ratio differences between all three sample classes were then determined as described in the Methods section since the data were shown not to be normally distributed (Figure 5). The results showed statistical significance at a 5% significance level for both NMNA and NMND between autistic and control individuals. NMNA, NMND and succinate were found to be significant between siblings and autistic individuals in the pair-wise comparison (Figure 5). Although we observed a trend towards decreased excretion of hippurate, 4-cresol sulfate and

phenylacetylglutamine in the autistic group due to a high degree of variability, possibly reflecting multiple sub-phenotype for the autism group, the t-test results for these metabolites were not statistically significant

Discussion

The results of this study have shown significant differences in the metabolic composition of urine between children with autism and unrelated non autistic controls. Statistical analysis of the urinary NMR data using O-PLS-DA coefficients, permutation testing or univariate t-test on the ratios of selected metabolites to creatinine, indicated that children with autism have lower levels of urinary hippurate and PAG, and higher levels of urinary DMA. More importantly, this study showed that children with autism demonstrate systematic metabolic differences as compared to normal controls. Pattern recognition analyses indicated that the main metabolic changes were in concentrations of mammalian-microbial co-metabolites such as DMA, hippurate, PAG, and 4-cresol sulfate and in nicotinic acid metabolism as evidenced by changes in the levels of 2PY, NMNA and NMND. Additionally amino acids such as taurine and glutamate, NAG and succinate were consistently altered in the ASD group. Additionally, from univariate t-tests on the ratios of selected metabolites to creatinine analysis, NMNA, NMND and succinate were found to be significantly different between the siblings and the controls, which indicated that the siblings were also different from the controls although multivariate analysis did not reflect this changes.

Gut microbial/host co-metabolite differences. Changes in the levels of urinary aromatic compounds such as 4-cresol sulfate, hippurate and PAG suggested the involvement of gut microflora in autism, since their precursors, benzoic acid, and phenylacetic acid respectively, are produced by bacterial metabolism in the intestine.³¹⁻³⁴ Hippurate is predominantly formed by hepatic glycine conjugation of dietary and gut microbial-derived benzoate, which is derived from plant phenolics.³³ Here, the decreased urinary levels of hippurate is suggestive of reduced benzoic acid synthesis. Protein-derived aromatic amino acids such as phenylalanine and tyrosine are also catabolized by host gut microbiota to form

PAG and 4-cresol sulfate.^{31, 34} Gut microbiota have been shown to facilitate host energy recovery from dietary sources by providing refined control mechanisms on energy recovery through catabolism of otherwise poorly digestible nutrients e.g. resistant starch.³⁵ Changes in the concentrations of urinary hippurate and PAG have been previously linked to the effects of xenobiotics on the intestinal microbial metabolism.³⁶ Depletion of hippurate and PAG has been reported after ingestion of antibiotics such as vancomycin¹² and gentamicin.³⁷ In addition, gastrointestinal microbial analysis of fecal samples from children with late-onset autism has shown that autistic individuals have higher incidence of the *Clostridium histolyticum* group of bacteria, as well as increased diversity of clostridial species^{11, 13}, and individuals with autism have been shown to have abnormalities in hippurate excretion.³⁸ Abnormalities of the gut microbiota including the Clostridium sp. a group of bacteria with a wide range of species variation in host hostility have also been noted in autistic children.¹¹ Many species of clostridia can produce powerful neurotoxins, and these have been implicated in ASD with certain clostridial species being found to be specific to autistic individuals (and not present in non autistic children).¹³ The fecal microbiota of ASD individuals have been found to contain higher numbers of the *Clostridium histolyticum* group of bacteria in comparison with to healthy children. Their non-autistic siblings were found to have intermediate levels of the same group of bacteria indicating that the importance of gastrointestinal microbiota composition in ASD may be influential in the development of the condition.¹¹ However, further metagenomic studies on the fecal samples of these children will be required to confirm the exact role of the gut microbiota species variation. The changes in urinary mammalian-microbial co-metabolites, observed in the current study, highlight the potential involvement of gut microbiota in autism and warrant further investigation of the effect of gut microbe metabolites on early brain function development.

Endogenous biochemical differences in autism: In the present study, urinary amino acids glutamate and taurine, were found to be perturbed in autistic

individuals as compared to the controls in the pattern recognition analyses. Several studies have shown that children with ASD often suffer from dysregulated amino acid metabolism.^{39, 40} In addition, Rolf *et al.* reported that platelet serotonin was significantly increased and amino acids aspartate, glutamine, glutamate and γ -aminobutyrate were significantly decreased in autistic individuals compared to the controls⁴¹ and Arnold *et al.* found significantly lower levels of glutamine in 12 autistic children compared to age-matched controls.⁴² The increase in urinary NAG in the autistic group could indicate the involvement of autoimmunity in autism since increased excretion of NAGs in serum and urine have been associated with inflammatory conditions^{43, 44} and autoimmunity has been implicated in autistic children.⁴⁵ The changes in urinary succinate in the current study suggested a change in energy metabolism since succinate plays a biochemical role in the citric acid cycle. However, it is likely that the change observed in succinate is related to the perturbation in amino acid metabolism as observed in the current study.

2PY, NMNA and NMND, metabolites of nicotinic acid metabolism, were found to be increased in individuals with autism, and were the group of metabolites with the greatest discriminating power between autistics and age-matched controls by the univariate analyses. The differences observed in these metabolite concentrations cannot be explained simply by intake of exogenous compounds since the samples used in the data analysis were pre-selected from individuals, who were reported not to have taken vitamin supplements. These metabolites are end-products of nicotinamide metabolism, which is derived from tryptophan. Nicotinamide is involved in the tryptophan-NAD pathway that supplies pyridine nucleotides to the liver. Nicotinamide is metabolized to NMND via nicotinamide *N*-methyltransferase and subsequently to 2PY and *N*-methyl-4-pyridone-3-carboxamide (4PY) *via* the action of aldehyde oxidase.^{46, 47} Nicotinic acid metabolism and high excretion of NMND has been implicated in Parkinson's disease.⁴⁸⁻⁵⁰ NMND has been shown to have *N*-methylated pyridines-like dopaminergic toxicity.⁵¹ NMND has also been demonstrated to destroy cerebral complex 1 subunits⁵² and complex 1 deficiency plays a role in the pathogenesis

of Parkinson's disease.⁵³⁻⁵⁵ The changes in urinary 2PY, NMNA and NMND observed in the current study suggested possible perturbation of tryptophan-nicotinic acid metabolism in autistic individuals.

Sleep disorders are common in autism and melatonin supplementation is used for treatment.⁵⁶ Perturbation in tryptophan-serotonin-melatonin pathways has also been implicated in autism.^{57, 58} Tryptophan acts as a biochemical precursor for both melatonin and nicotinic acid. The increases in the metabolites of nicotinic acid metabolism would indicate an increase in tryptophan metabolism towards the formation of nicotinic acid. This can be due to reduced enzyme activity converting tryptophan to serotonin and subsequently to melatonin. The conversion of tryptophan to melatonin involves two enzymes, tryptophan hydroxylase and acetylserotonin methyltransferase (ASMT).⁵⁹ Autistic individuals have been shown to have reduced ASMT enzyme activity.⁵⁹ Therefore, a decrease in conversion of tryptophan to melatonin could consequently lead to an increase in nicotinic acid synthesis as reflected in this study. Melatonin was not observed in the current study because of its expected low urinary concentration, below the limit of NMR detection.

Autistic children are known to suffer from a decreased capacity for methylation and vulnerability to oxidative stress.^{60, 61} James et al (2004) measured plasma concentrations of methionine, S-adenosylmethione (SAM), S-adenosylhomocysteine (SAH) and other sulfur-containing metabolites and found that autistic children have significantly lower plasma concentration of methionine, SAM (an important methyl donor), homocysteine and total glutathione relative to control children.⁶⁰ An increased demand for methylation of nicotinic acid to its *N*-methylated acid and amide as observed in our study would exert further stress on what is already a compromised methylation capacity in autistic children leading to increased susceptibility to oxidative stress.

The increase in urinary taurine in the autistics could be indicative of alteration in cysteine metabolism in autistic individuals. Taurine levels were found to be significantly increased in autistic children as evidenced by the results of the permutation test. However, taurine concentrations were hypervariable in the

autistic group, which did not detect any difference between groups based on t-test analysis. Nevertheless, this hypervariability is in itself interesting and may indicate the presence of more than one phenotype associated with autism. As noted above, oxidative stress has been suggested to contribute to the development of autism.⁶⁰⁻⁶³ Taurine plays an important role in cellular protection against free radical and oxidative stress. Children with autism have been shown to have low plasma levels of inorganic sulfate and sulfur oxidation deficiencies.¹⁴ In a follow-up study, Waring and Klovza showed that urinary excretion of sulfate, sulfite and thiosulfate were increased in autistic children further supporting perturbation in sulfur metabolism.¹⁴ They postulated that an increase in urinary sulfur indicated dysfunction in specific sulfate transporters, NaSi and SAT-1.¹⁴ These transporters are involved in transporting sulfate across the apical and basolateral membranes of the renal tubule cells thus playing an important role in maintaining body supplies of sulfur and sulfate. The increase in urinary taurine observed in this study is further evidence that these children may suffer from a defect in sulfate transporters and abnormal sulfur metabolism. Indeed it is known that autistic children have an inability to sulfate phenolic compounds and drugs such as acetaminophen,¹⁴ which also implicates a fundamental problem with sulfur dependent detoxification reactions. Reduced capacity to sulfate acetaminophen has also recently been noted in normal humans with high pre-dose 4-cresol levels,⁶⁴ which is a compound produced by selected clostridia, which are themselves abnormal in autistic children.¹³ We have recently shown that even in normal individuals high levels of gut microbially produced 4-cresol can compromise sulfation of acetaminophen at therapeutic doses, and further that *N*-acetyl cysteinyl and cysteinyl adducts of acetaminophen are lower in urines of people with high levels of pre-dose 4-cresol sulfate.⁶⁴ This suggests a generalized depletion of sulfur metabolism (including the glutathione pathway) and a consequent inability to detoxify reactive intermediates and this may have long term significance in autistic patients. The possible connections between abnormal microbial metabolic activity and abnormal sulfur metabolism in the host and whether these have any etiologic or developmental significance are

intriguing possibilities but require further investigation in longitudinal developmental studies.

Conclusions

This work has characterized a series of urinary metabolic changes that are associated with autism. Specifically, the metabolic profiling approach also indicated changes in gut microbiota metabolism, amino acid metabolism and nicotinic acid metabolism in autistic children. The excretion patterns of a panel of urinary metabolites *viz* hippurate, NMNA, NMND and taurine were abnormal and these now need further validation as potential biomarkers for autism. Also, whether the metabolic differences are related to the causes of the condition or are a consequence of the progression of the disease, remains to be determined in larger scale longitudinal studies. It should be noted however that biomarkers of any established disease condition are not necessarily the same as those occurring during the disease etiology and that mechanistic understanding would be further enhanced by studying larger groups of early onset patients much closer to the time of diagnosis. Nevertheless, further investigation of the metabolic pathways that we find to be perturbed in the present study could provide new clues to the etiology of autism and give rise to novel criteria for therapeutic interventions.

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Figure legends

Figure 1. (A) Median 600 MHz ^1H NMR spectra of urine from the groups of control, sibling and autistic individuals. The spectral region of nicotinic acid derivatives (δ 8.6-9.5) is shown in the inset.

Key: 2PY*, tentatively assigned as *N*-methyl-2-pyridone-5-carboxamide; DMA, dimethylamine; DMG, dimethylglycine; NMNA, *N*-methyl nicotinic acid; NMND, *N*-methyl nicotinamide; TMA, trimethylamine; TMAO, trimethylamine *N*-oxide; PAG, phenylacetylglutamine; 4CS, 4-cresol sulfate;

Figure 2. (A) PCA scores plot of the first 2 components from the normalized UV-scaled NMR data and (B) the corresponding PLS-DA cross-validated scores plot for all three group. PLS-DA cross-validated scores plot of pairwise comparison between (C) controls versus siblings and (D) controls versus autistics.

Figure 3. O-PLS-DA coefficients plot showing differences in urinary profiles between controls and autistics.

Figure 4. Covariance plot showing the significance of the urinary profiles calculated using the permutation test between control and autistics.

Figure 5. The ratio of selected urinary metabolites to that of urinary creatinine across the 3 groups.

Key: *, ***, indicates a significant difference at $p < 0.05$, 0.001 confidence levels respectively.

Figure 1

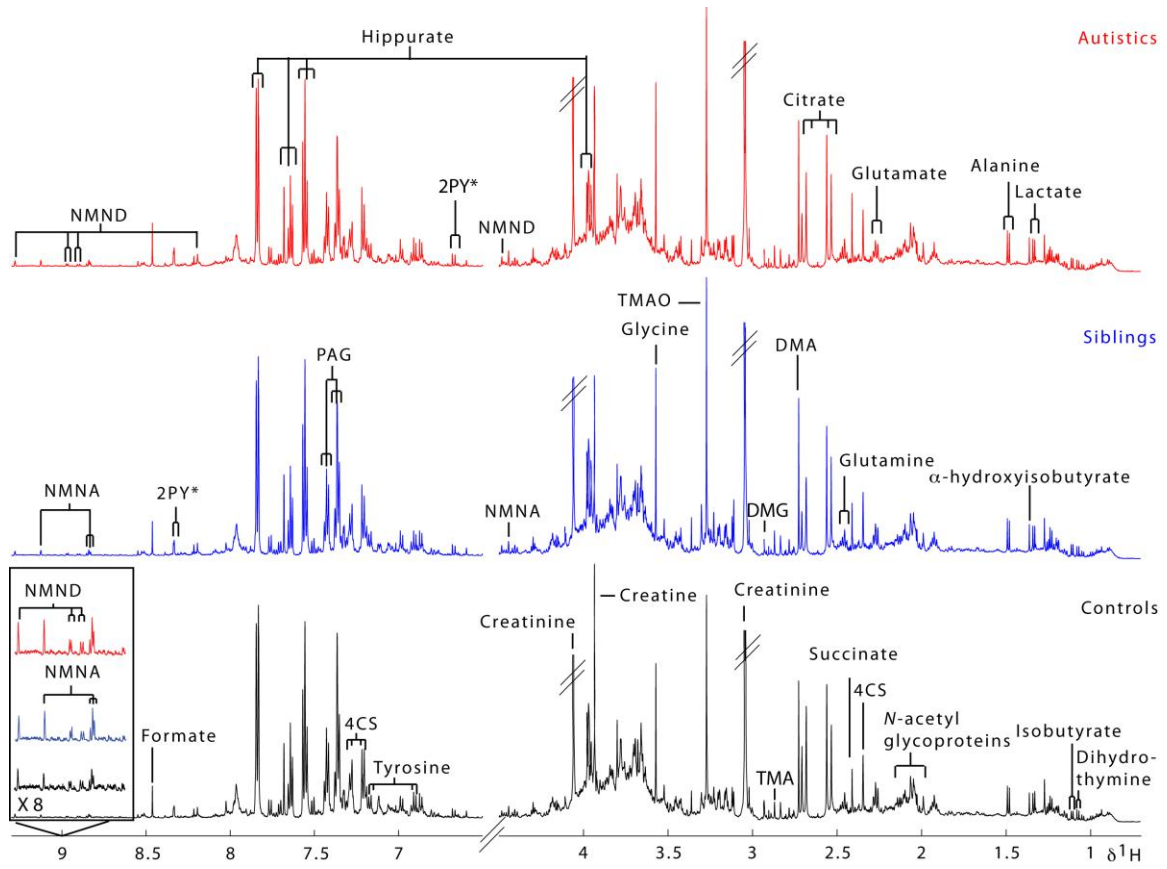


Figure 2

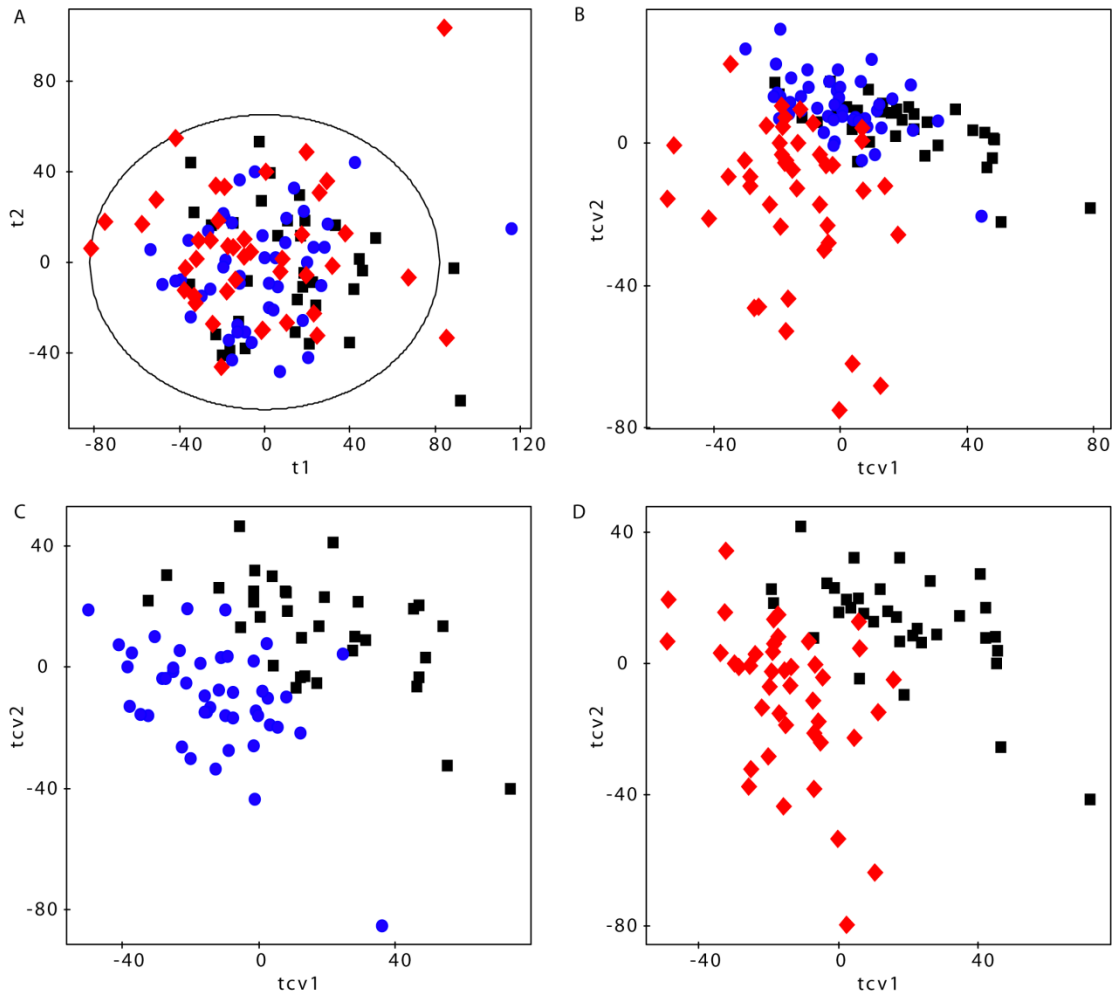


Figure 3

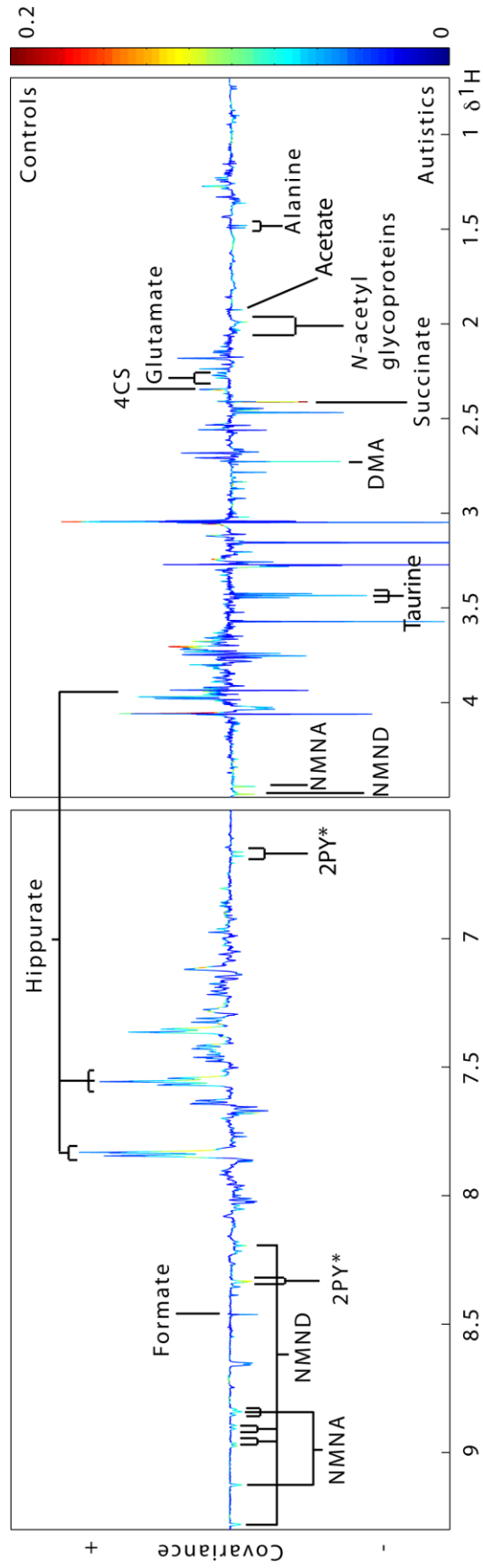


Figure 4

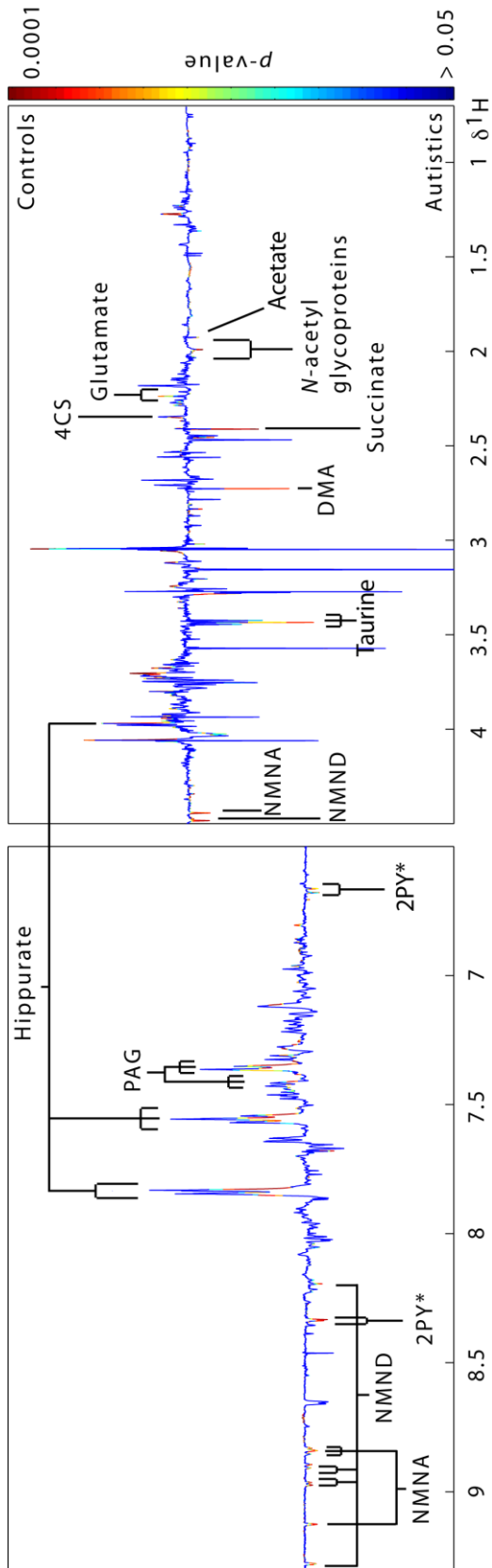
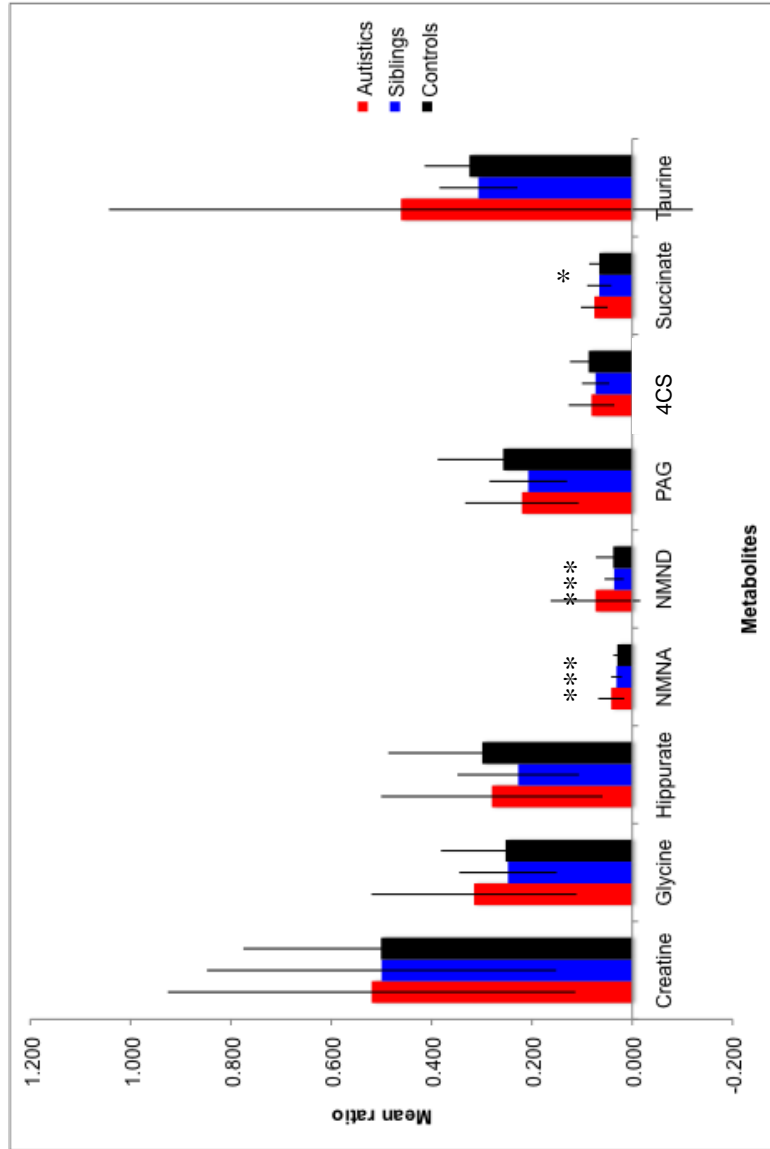
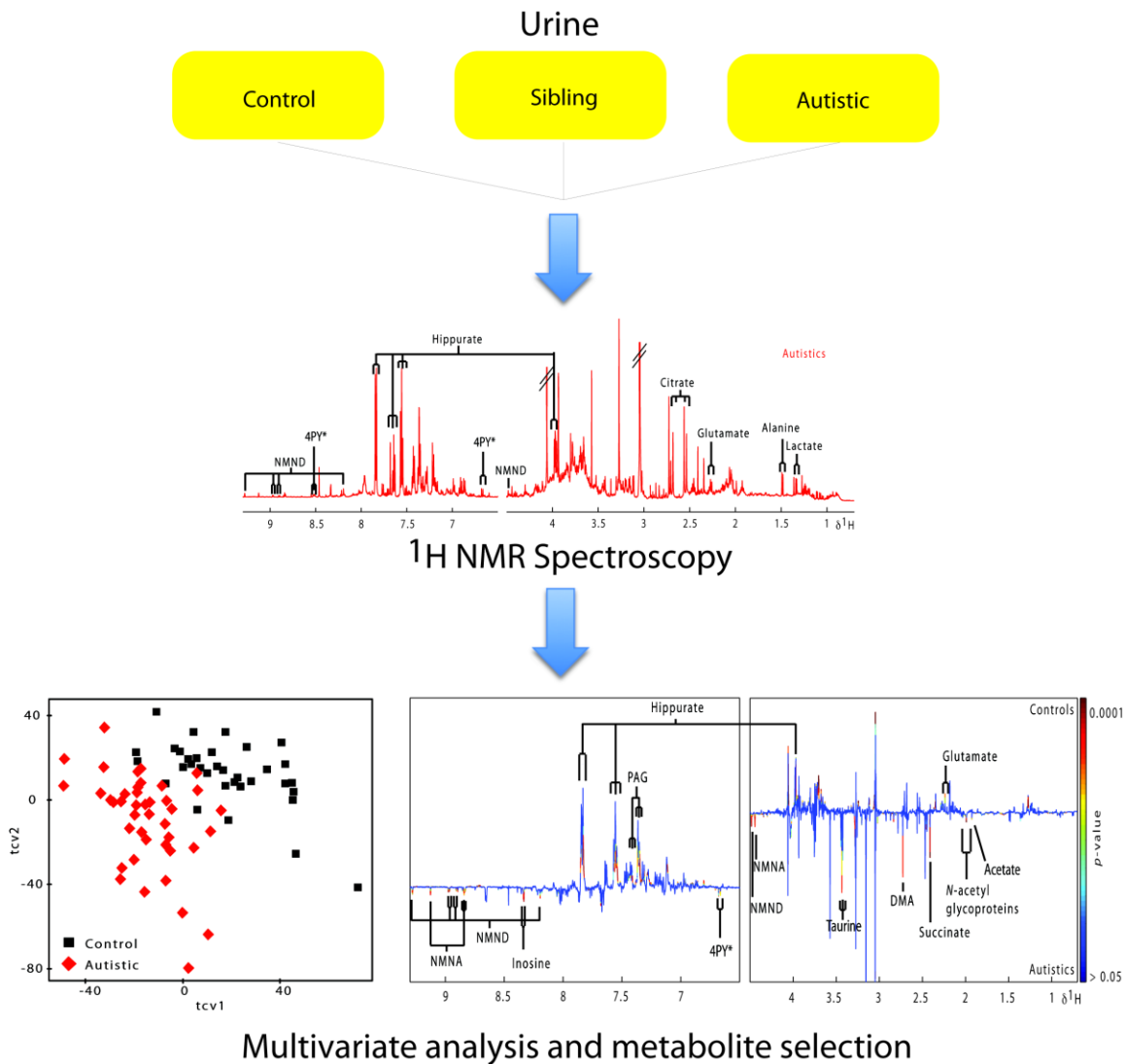


Figure 5



Synopsis



Autism is an early-onset developmental disorder with complex neurodevelopmental and socio-psychological problems associated with metabolic abnormalities. A suite of metabolic changes that are associated with autism were characterized using NMR-based metabonomic and show systematic differences in the urinary composition of autistic children with respect to their non-symptomatic siblings and age matched controls. Several metabolic pathways were shown to be affected by autism in this study, which opens new directions for validation of potential biomarkers.