

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-4-pyridone-3-carboxamide, *N*-methyl nicotinic acid and *N*-methyl nicotinamide, indicate a perturbation in tryptophan-nicotinic acid metabolic pathway. Urinary patterns of the free amino acids glutamate, alanine, glycine and taurine were significantly different between groups with the autistic children showing higher levels of urinary alanine, glycine and 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 of urinary mammalian-microbial co-metabolites including dimethylamine, hippurate, phenylacetylglutamine and 4-cresol sulfate. 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

Over the years 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

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3 in 10 000 in 2006 in the United States¹⁶ alone, but this varies regionally and with
4 ethnicity, and also some geographically localized areas have much higher
5 incidences of ASD.¹⁷ However, it is not clear whether the global increase is due
6 to higher prevalence of the disorder, and/or improved early detection/diagnosis.
7 Current diagnosis of ASD is subjective and depends on observations of a cluster
8 of behaviors and fulfillment of multiple criteria set out in the Diagnostic and
9 Statistical Manual of Mental Disorders 4th edition (DSM-IV-TR)¹⁸ by a trained
10 clinician. At present, there are no reliable biochemical- or genetic-screening tests
11 for the disorder and in some cases, particularly in late onset autism, childhood
12 development can switch from being normal to showing a delay in acquisition of
13 new skills, thus adding to the difficulty for diagnosing ASD. Thus, there is a
14 pressing need for new diagnostic tools for ASD that are both sensitive and
15 reliable, since early diagnosis can lead to timely interventions and optimized
16 clinical management.
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28 Metabonomic approaches offer the possibility of measuring metabolic end
29 points (metabolic profiles) that are determined by host genetic and environmental
30 factors.^{19, 20} The application of high throughput metabolic profiling methods using
31 high resolution analytical platforms (nuclear magnetic resonance (NMR)
32 spectroscopy and/or mass spectrometry (MS)) with subsequent multivariate
33 statistical analyses now provides a well-established strategy for differential
34 metabolic pathway profiling and disease diagnosis.^{10, 20-22} Here we apply a
35 metabolic profiling approach to capture the global biochemical signature of
36 autistic individuals using NMR spectroscopy with multivariate statistical modeling
37 to characterize individuals with autism, unaffected siblings and unrelated control
38 healthy comparison groups in population-based samples. The key differential
39 metabolic features of autism derived from the current study can subsequently be
40 evaluated as candidate diagnostic markers in further studies.
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52 **Methods**

53 **Sample Collection.** The urine samples used in this study were obtained from
54 University of South Australia (autistic subjects, their siblings and unrelated
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3 healthy subjects; age range: 3-9 years old) and a second group of controls from
4 the Swiss Tropical Institute (comparable healthy subjects; age range: 3-9 years
5 old), and complied with Australian and Swiss local and national regulations on
6 ethics.
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10 In order to address bias deriving from geographical location, urine samples from
11 the second group of controls were profiled together with the first group of
12 controls. The two control data sets were shown to be statistically
13 indistinguishable (data not shown). Each individual and their family gave
14 informed consent for the study to take place. Autistic participants were diagnosed
15 according to the DSM-IV-TR criteria for diagnosis of autism disorder or Asperger
16 syndrome.¹⁸ Only children diagnosed to be autistic are included in this study.
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20 **Sample Preparation.** Urine samples were prepared by mixing 400 μ l of urine
21 with 220 μ l of a phosphate buffer (90% D₂O, 1 mM 3-trimethylsilyl-1-[2,2,3,3-²H₄]
22 propionate (TSP), and 3 mM sodium azide; pH 7.4) and left to stand for 10 min.
23 The samples were centrifuged at 11000 g for 10 min and 600 μ l of the
24 supernatant for each sample was then transferred into 5 mm (outer diameter)
25 NMR tubes.
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29 **¹H NMR Spectroscopy.** Spectra were obtained on a Bruker Avance600
30 spectrometer (Bruker Biospin; Rheinstetten, Germany) at 600.13 MHz (ambient
31 probe temperature 27°C). A standard 1-dimensional (1D) pulse sequence was
32 used [recycle delay (RD)-90°- t_1 -90°- t_m -90°-acquire free induction decay (FID)].
33 The water signal was suppressed by irradiation during the RD of 2 s, the mixing
34 time (t_m) of 150 ms. t_1 was set to 3 μ s and the 90° pulse length was ~10 μ s. For
35 each sample, a total of 128 transients were accumulated into 32k data points
36 using a spectral width of 20 ppm. Prior to Fourier transformation, all FIDs were
37 multiplied by an exponential function equivalent to a line broadening of 0.3 Hz.
38 The assignment of the peaks to specific metabolites was based on 2-dimensional
39 (2D) ¹H-¹H correlation spectroscopy (COSY) and ¹H-¹H total correlation
40 spectroscopy (TOCSY) NMR spectra, published literature,^{23, 24} and statistical
41 total correlation spectroscopy (STOCSY).²⁵
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3 **Data Processing and Analysis.** ^1H NMR spectra of urine samples were
4 manually phased and baseline corrected using XwinNMR 3.5 (Bruker Biospin;
5 Rheinstetten, Germany). The ^1H NMR spectra were referenced to the TSP
6 resonance at δ 0.0. Spectra were digitized using a MATLAB (version 7, The
7 Mathworks Inc.; Natwick, MA, USA) script developed in-house. The regions
8 containing the water and urea resonances (δ 4.5 – 6.5) were removed from each
9 spectrum to eliminate baseline effects of imperfect water saturation. For each
10 spectrum, a recursive peak alignment algorithm²⁶ was applied to minimize
11 spectral peak shifts due to residual pH differences within samples prior to
12 normalization to the total area sum of the residual spectrum and subsequent
13 pattern recognition analyses.
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23 Principal components analysis (PCA) was applied to the unit variance
24 (UV)-scaled spectral data to reveal intrinsic autism-related patterns. Projection to
25 latent structure discriminant analysis (PLS-DA) with UV-scaled spectral data was
26 also carried out in order to improve classification of the different groups of
27 individuals as well as to optimize the identification of changes in urinary
28 metabolites that were unique to a particular group. Permutation testing (200
29 permutations of the class parameters) was performed on the PLS-DA models to
30 ensure statistical model validity. Inter-individual variation can confound data
31 interpretation, particularly in multivariate data of high dimensionality. Therefore,
32 orthogonal projection to latent structure discriminant analysis (O-PLS-DA)²⁷ was
33 also performed on UV-scaled spectral data to optimally model class differences
34 and to systematically identify metabolites contributing to the differences between
35 autistic, siblings and control groups by removing systematic variation in the
36 profiles that does not relate to autism, thereby enhancing the interpretability of
37 the models. Back scaling transformation of the O-PLS coefficients to the
38 covariance matrix was also used to increase the interpretability of the model.²⁸
39 Here, the colors projected onto the coefficient plot indicate the correlation of the
40 metabolites discriminating each class from the corresponding controls. Red
41 indicates a high correlation and blue denotes no correlation with sample class.
42 The direction and magnitude of the signals relate to the covariation of the
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3 metabolites with the classes in the model and the model predictive performance
4 (robustness) was evaluated using a 7-fold cross validation method as defined by
5 the Q^2 (goodness of prediction) parameter.²⁸ In addition, the statistical
6 significance and validity of the O-PLS-DA results were calculated using a
7 permutation test (number of permutations = 10000).^{29, 30} The significance of each
8 metabolite from the permutation test is interpreted using the covariance plot,
9 where the colors projected onto the spectrum indicate the significance of the
10 metabolites with blue indicating no significance difference at $p > 0.05$ confidence
11 levels and red indicating high significance difference at $p < 0.0001$; the direction
12 and magnitude of the signals relate to the covariation of the metabolites with the
13 classes in the model.
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22 **Calculation of ratios of selected urinary metabolites to creatinine.** The
23 relative mean concentrations of a range of urinary metabolites such as 4-cresol
24 sulfate (δ 2.35), creatine (δ 3.93), glycine (δ 3.56), hippurate (δ 7.84), *N*-methyl
25 nicotinic acid (NMNA) (δ 4.45), *N*-methyl nicotinamide (NMND) (δ 4.48),
26 phenylacetylglutamine (PAG) (δ 7.43), succinate (δ 2.41) and taurine (δ 3.43)
27 were calculated from the peak area of the designated NMR signals for the
28 metabolites listed above using a MATLAB script developed in-house to partially
29 overcome any concentration effects caused by different urine volumes. The
30 ratios of the peak areas of these selected metabolites to that of creatinine (δ
31 4.04) were then calculated and a non-parametric 2-tailed Mann-Whitney test was
32 performed to calculate the significance level of class differences based on the
33 ratios between groups.
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46 Results

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48 **Urinary ^1H NMR spectroscopic profiles of the cohort.** Urinary 600 MHz ^1H
49 NMR median spectra of individuals from the 3 groups (Figure 1) contained peaks
50 from a wide range of low-molecular-weight metabolites of diverse chemical
51 classes (typically <1 kDa) from both mammalian and associated gut-microbial
52 metabolism. The urinary NMR spectra were dominated by dietary and microbial-
53 derived methylamines (dimethylamine (DMA), trimethylamine *N*-oxide (TMAO))
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3 and aromatic substances such as hippurate, phenylacetylglutamine (PAG) and 4-
4 cresol sulfate (4-cresol sulfate) (Figure 1). Additionally, mammalian metabolites
5 such as citrate, succinate, creatinine, lactate, α -hydroxyisobutyrate and amino
6 acids (particularly alanine, glutamine and glycine) were also apparent. Visually,
7 allowing for the inter-individual variability, the urinary spectra were very similar,
8 but the autistic individuals showed subtle differences in urinary succinate, *N*-
9 methyl nicotinic acid (NMNA) and *N*-methyl nicotinamide (NMND) compared to
10 the controls, as evidenced from the median spectra shown in Figure 1.

11
12 **Multivariate statistical analysis of the NMR spectral data.** To further explore
13 the metabolic differences between the three groups of participants, multivariate
14 statistical analyses were employed on the full resolution NMR data set consisting
15 of 34 controls, 28 siblings and 39 autistic urine samples to extract useful
16 metabolic information. PCA was carried out on UV-scaled data to identify any
17 inherent differences within the dataset. The resulting scores plot of PC1 versus
18 PC2 (Figure 2A) showed no clear differences between the three groups, nor did
19 examination of all pairs of lower PCs up to 3 PCs provide any discrimination
20 indicating that the major source of variation in the data was not related to autism,
21 but was rather dominated by inter-person variability. However, by utilizing group
22 information in PLS-DA analysis, systematic differences could be observed
23 between the three groups (Q^2 : 15%; R^2 (goodness of fit): 65.7%). The
24 corresponding cross-validated PLS-DA scores plot (Figure 2B) showed clear
25 separation between autistic individuals and the controls and partial separation
26 between siblings and the controls. PLS-DA pair-wise comparison between
27 controls and siblings (Figure 2C; Q^2 : 31.4%, R^2 : 86%), and between controls and
28 autistics (Figure 2D; Q^2 : 33.5%, R^2 : 72%) suggested a robust metabolic
29 difference between the classes in each pair-wise comparison (Figure 2C and D
30 respectively) on the first component.

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32 To minimize any effects of non-relevant metabolite variability, pair-wise O-
33 PLS-DA analyses were carried out on the UV-scaled NMR data set to identify
34 urinary metabolites contributing to the differences between groups. Systematic
35 comparisons of the autistics and normal control groups using O-PLS-DA
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3 generated a model with a Q^2 of 15% and R^2 of 27.5%. The corresponding
4 coefficients plot (Figure 3) indicated differences in the urinary metabolic profile
5 between the two groups with the autistics showing relatively higher levels of
6 urinary acetate, DMA, *N*-acetyl glycoprotein fragments (NAG), glycine, succinate,
7 alanine, taurine, formate, inosine, NMNA, NMND and *N*-methyl-4-pyridone-3-
8 carboxamide (4PY), whilst the healthy controls showing higher levels of urinary
9 glutamate, hippurate, PAG, and 4-cresol sulfate. Comparison between siblings
10 and the controls, and between siblings and autistics, using O-PLS-DA, showed
11 no differences in the urinary metabolic profile (data not shown) and the model
12 generated was very weak (negative Q^2). Permutation tests were carried out on
13 the pair-wise area-normalized NMR data to statistically validate the results
14 obtained from the O-PLS-DA analyses. Pair-wise comparison between the
15 controls and autistic individuals showed statistical significance at a $p < 0.01$
16 significance level (Figure 4) on all metabolites identified to be contributing to the
17 differences between controls and autistics in the O-PLS-DA analyses (Figure
18 3A). However, permutation tests carried out on the controls and siblings failed to
19 find statistical significance in the urinary metabolites (data not shown), which is
20 reflective of the weak Q^2 and R^2 values generated from the O-PLS-DA model.

21 **Ratios of selected urinary metabolite concentrations against creatinine.**

22 The areas of selected NMR peaks from a range of urinary metabolites
23 determined to be significantly different between groups *via* multivariate statistical
24 modeling, such as creatine, creatinine, glycine, hippurate, NMNA, NMND, PAG,
25 4-cresol sulfate, succinate and taurine were quantitated and ratios obtained
26 relative to that of the methylene peak of creatinine. The significance of the ratio
27 differences between all three sample classes were then determined as described
28 in the Methods section since the data were shown not to be normally distributed
29 (Figure 5). The results showed statistical significance at a 5% significance level
30 for both NMNA and NMND between autistic and control individuals. NMNA,
31 NMND and succinate were found to be significant between siblings and autistic
32 individuals in the pair-wise comparison (Figure 5).
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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. Multivariate statistical analysis of the urinary NMR data indicated that children with autism have lower levels of urinary hippurate and PAG, and higher levels of urinary glycine and 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 4PY, NMNA and NMND. Additionally amino acids such as alanine and glycine, NAG, succinate, taurine and glutamate were consistently altered in the ASD group.

Gut microbial/host co-metabolite differences. Changes in the levels of urinary aromatic compounds such as 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 and increased glycine are suggestive of either reduced glycine conjugation or 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

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3 shown that autistic individuals have higher incidence of the *Clostridium*
4 *histolyticum* group of bacteria, as well as increased diversity of clostridial
5 species^{11, 13}, and individuals with autism have been shown to have abnormalities
6 in hippurate excretion.³⁸ Abnormalities of the gut microbiota including the
7 *Clostridium* sp. a group of bacteria with a wide range of species variation in host
8 hostility have also been noted in autistic children.¹¹ Many species of clostridia
9 can produce powerful neurotoxins, and these have been implicated in ASD with
10 certain clostridial species being found to be specific to autistic individuals (and
11 not present in non autistic children).¹³ The fecal microbiota of ASD individuals
12 have been found to contain higher numbers of the *Clostridium histolyticum* group
13 of bacteria in comparison with to healthy children. Their non-autistic siblings were
14 found to have intermediate levels of the same group of bacteria indicating that
15 the importance of gastrointestinal microbiota composition in ASD may be
16 influential in the development of the condition.¹¹ However, further metagenomic
17 studies on the fecal samples of these children will be required to confirm the
18 exact role of the gut microbiota species variation. The changes in urinary
19 mammalian-microbial co-metabolites, observed in the current study, highlight the
20 potential involvement of gut microbiota in autism and warrant further investigation
21 of the effect of gut microbe metabolites on early brain function development.
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39 **Endogenous biochemical differences in autism:** In the present study, urinary
40 amino acids alanine and glycine, were found to be elevated in autistic individuals
41 as compared to the controls in the pattern recognition analyses. The increase in
42 urinary glycine in autistic individuals could be linked to a decrease in glycine
43 conjugation to benzoate in the liver to form hippurate, since autistic individuals
44 showed reduced levels of urinary hippurate in the current study. Alternatively,
45 several studies have shown that children with ASD often suffer from dysregulated
46 amino acid metabolism.^{39, 40} In addition, Rolf *et al.* reported that platelet serotonin
47 was significantly increased and amino acids aspartate, glutamine, glutamate and
48 γ -aminobutyrate were significantly decreased in autistic individuals compared to
49 the controls⁴¹ and Arnold *et al.* found significantly lower levels of glutamine in 12
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3 autistic children compared to age-matched controls.⁴² The increase in urinary
4 NAG in the autistic group could indicate the involvement of autoimmunity in
5 autism since increased excretion of NAGs in serum and urine have been
6 associated with inflammatory conditions^{43, 44} and autoimmunity has been
7 implicated in autistic children.⁴⁵

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12 4PY, NMNA and NMND, metabolites of nicotinic acid metabolism, were
13 found to be increased in individuals with autism, and were the group of
14 metabolites with the greatest discriminating power between autistics and age-
15 matched controls by the univariate analyses. The differences observed in these
16 metabolite concentrations cannot be explained simply by intake of exogenous
17 compounds since the samples used in the data analysis were pre-selected from
18 individuals, which reported not to have taken vitamin supplements. These
19 metabolites are end-products of nicotinamide metabolism, which is derived from
20 tryptophan. Nicotinamide is involved in the tryptophan-NAD pathway that
21 supplies pyridine nucleotides to the liver. Nicotinamide is metabolized to NMND
22 via nicotinamide *N*-methyltransferase and subsequently to *N*-methyl-2-pyridone-
23 3-carboxamide (2PY) and 4PY via the action of aldehyde oxidase.^{46, 47} Nicotinic
24 acid metabolism and high excretion of NMND has been implicated in Parkinson's
25 disease.⁴⁸⁻⁵⁰ NMND has been shown to have *N*-methylated pyridines-like
26 dopaminergic toxicity.⁵¹ NMND has also been demonstrated to destroy cerebral
27 complex 1 subunits⁵² and complex 1 deficiency plays a role in the pathogenesis
28 of Parkinson's disease.⁵³⁻⁵⁵ The changes in urinary 4PY, NMNA and NMND
29 observed in the current study suggested possible perturbation of tryptophan-
30 nicotinic acid metabolism in autistic individuals. Sleep disorders are common in
31 autism and melatonin supplementation is used for treatment.⁵⁶ Perturbation in
32 tryptophan-serotonin-melatonin pathways has been implicated in autism.^{57, 58}

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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

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3 melatonin involves two enzymes, tryptophan hydroxylase and acetylserotonin
4 methyltransferase (ASMT).⁵⁹ Autistic individuals have been shown to have
5 reduced ASMT enzyme activity.⁵⁹ Therefore, a decrease in conversion of
6 tryptophan to melatonin could consequently lead to an increase in nicotinic acid
7 synthesis as reflected in this study. Melatonin was not observed in the current
8 study because of its expected low urinary concentration, below the limit of NMR
9 detection.

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

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34 The increase in urinary taurine in the autistics could be indicative of
35 alteration in cysteine metabolism in autistic individuals. As noted above, oxidative
36 stress has been suggested to contribute to the development of autism.⁶⁰⁻⁶³
37 Taurine plays an important role in cellular protection against free radical and
38 oxidative stress. Children with autism have been shown to have low plasma
39 levels of inorganic sulfate and sulfur oxidation deficiencies.¹⁴ In a follow-up study,
40 Waring and Klovrsza showed that urinary excretion of sulfate, sulfite and
41 thiosulfate were increased in autistic children further supporting perturbation in
42 sulfur metabolism.¹⁴ They postulated that an increase in urinary sulfur indicated
43 dysfunction in specific sulfate transporters, NaSi and SAT-1.¹⁴ These
44 transporters are involved in transporting sulfate across the apical and basolateral
45 membranes of the renal tubule cells thus playing an important role in maintaining
46 body supplies of sulfur and sulfate. The increase in urinary taurine observed in
47 this study is further evidence that these children may suffer from a defect in
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3 sulfate transporters and abnormal sulfur metabolism. Indeed it is known that
4 autistic children have an inability to sulfate phenolic compounds and drugs such
5 as acetaminophen,¹⁴ which also implicates a fundamental problem with sulfur
6 dependent detoxification reactions. Reduced capacity to sulfate acetaminophen
7 has also recently been noted in normal humans with high predose 4-cresol
8 levels,⁶⁴ which is a compound produced by selected clostridia, which are
9 themselves abnormal in autistic children.¹³ We have recently shown that even in
10 normal individuals high levels of gut microbially produced 4-cresol can
11 compromise sulfation of acetaminophen at therapeutic doses, and further that *N*-
12 acetyl cysteinyl and cysteinyl adducts of acetaminophen are lower in urines of
13 people with high levels of pre-dose 4-cresol sulfate.⁶⁴ This suggests a
14 generalized depletion of sulfur metabolism (including the glutathione pathway)
15 and a consequent inability to detoxify reactive intermediates and this may have
16 long term significance in autistic patients. The possible connections between
17 abnormal microbial metabolic activity and abnormal sulfur metabolism in the host
18 and whether these have any etiologic or developmental significance are
19 intriguing possibilities but require further investigation in longitudinal
20 developmental studies.
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37 **Conclusions**

38 This work has characterized a series of metabolic changes that are associated
39 with autism. The metabolic profiling approach applied indicated changes in gut
40 microbiota metabolism, amino acid metabolism and nicotinic acid metabolism in
41 autistic children. The excretion patterns of a panel of urinary metabolites *viz*
42 hippurate, NMNA, NMND and taurine were abnormal and these now need further
43 validation as potential biomarkers for autism. Also, whether the metabolic
44 differences are related to the causes of the condition or are a consequence of the
45 progression of the disease, remains to be determined in larger scale longitudinal
46 studies. Nevertheless, further investigation of the metabolic pathways giving rise
47 to these substances could provide new clues to the etiology of autism and give
48 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: 4PY*, tentatively assigned as *N*-methyl-4-pyridone-3-carboxamide; DMA, dimethylamine; DMG, dimethylglycine; NMNA, *N*-methyl nicotinic acid; NMND, *N*-methyl nicotinamide; TMA, trimethylamine; TMAO, trimethylamine *N*-oxide; PAG, phenylacetylglutamine; PCS, 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 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

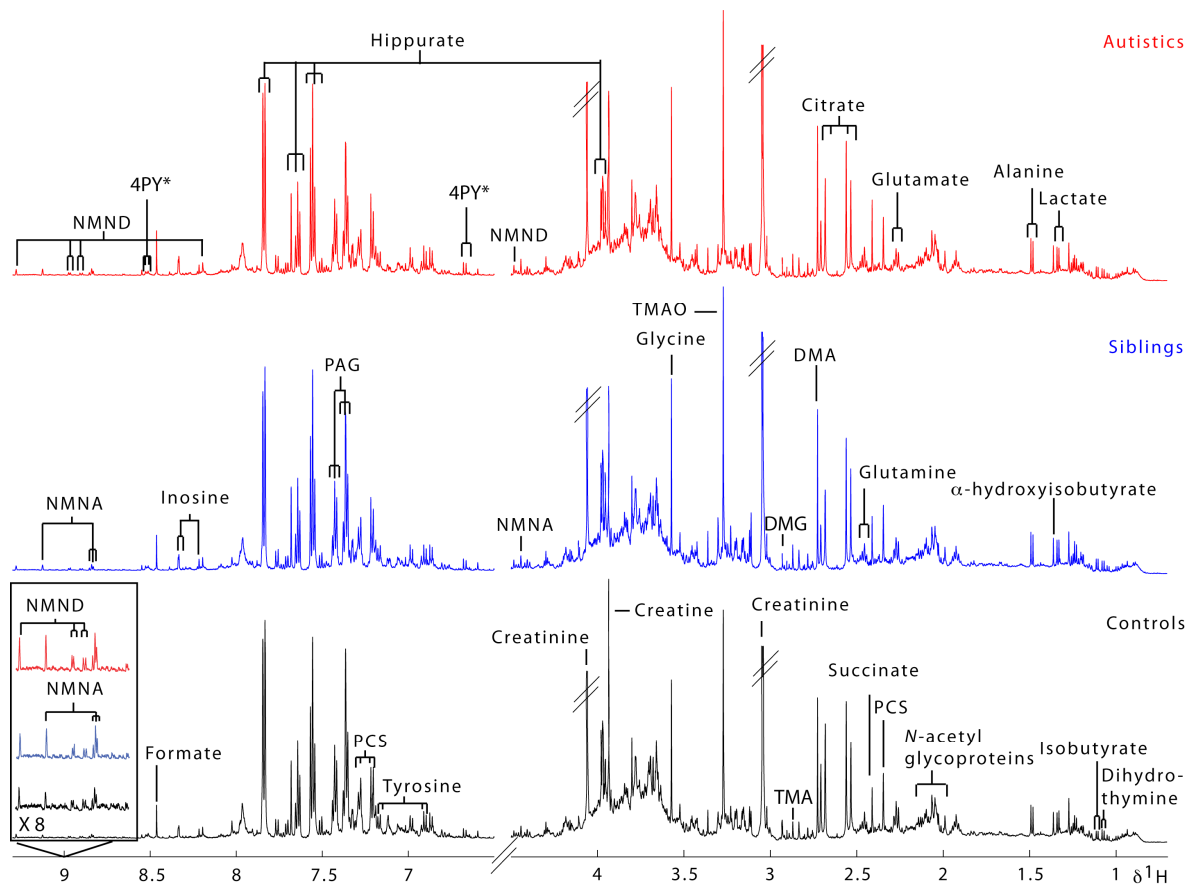


Figure 2

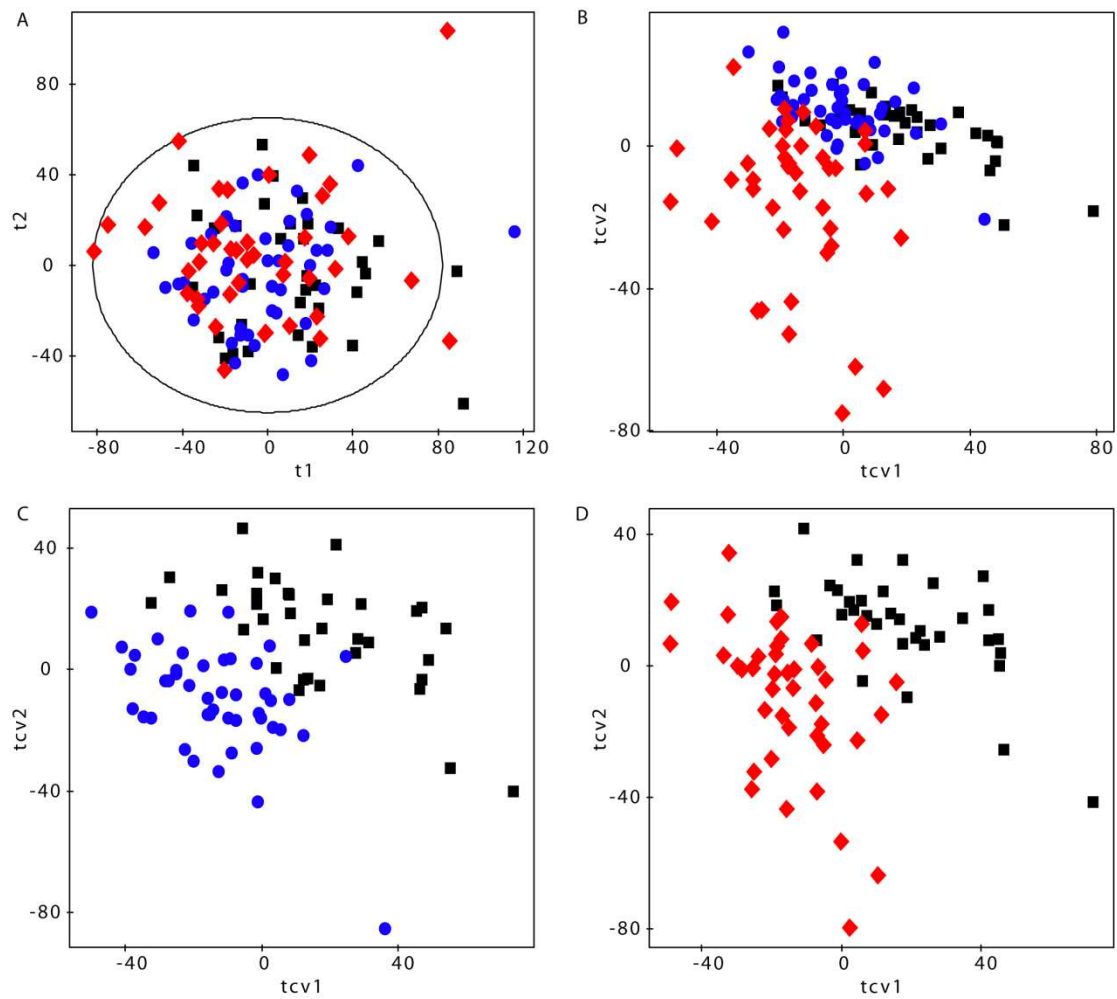


Figure 3

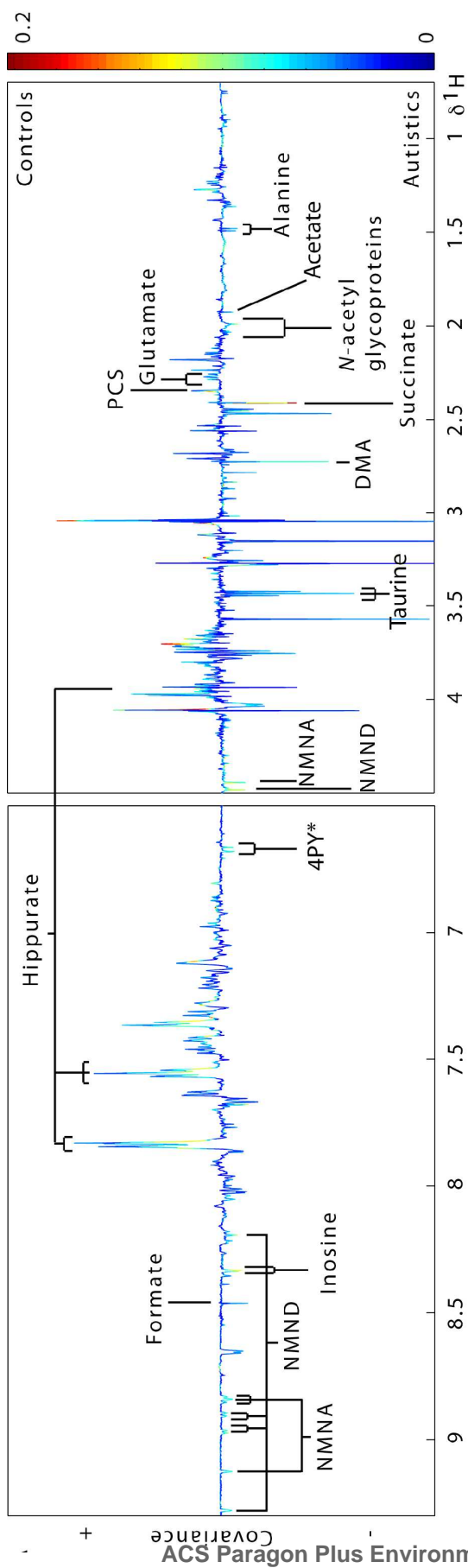


Figure 4

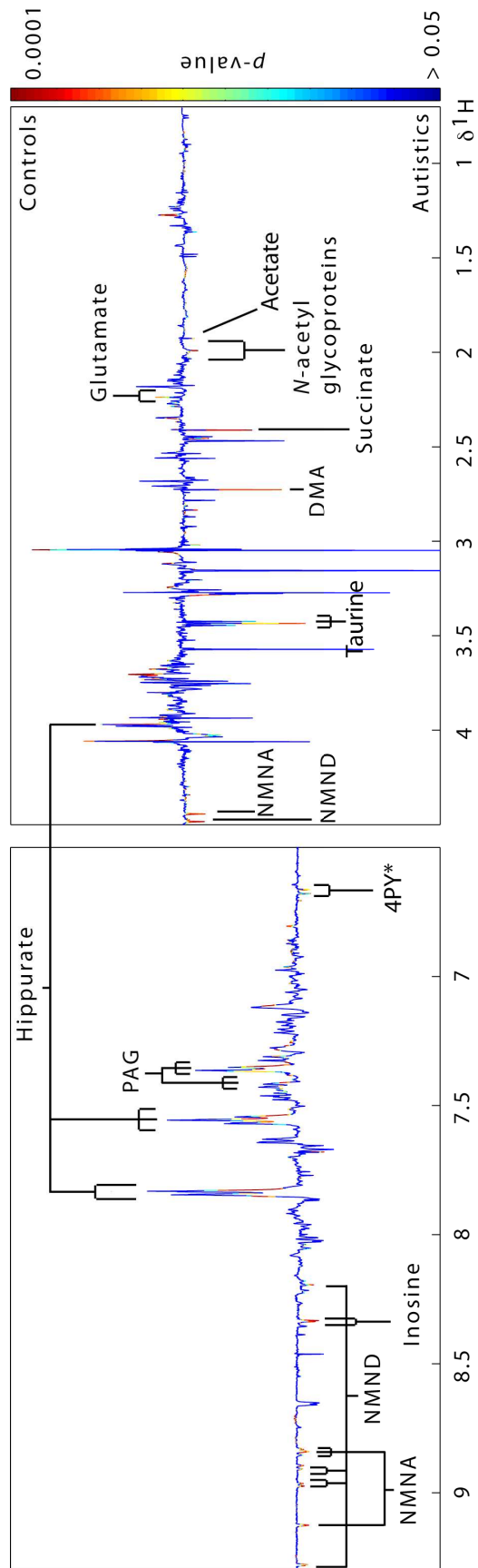
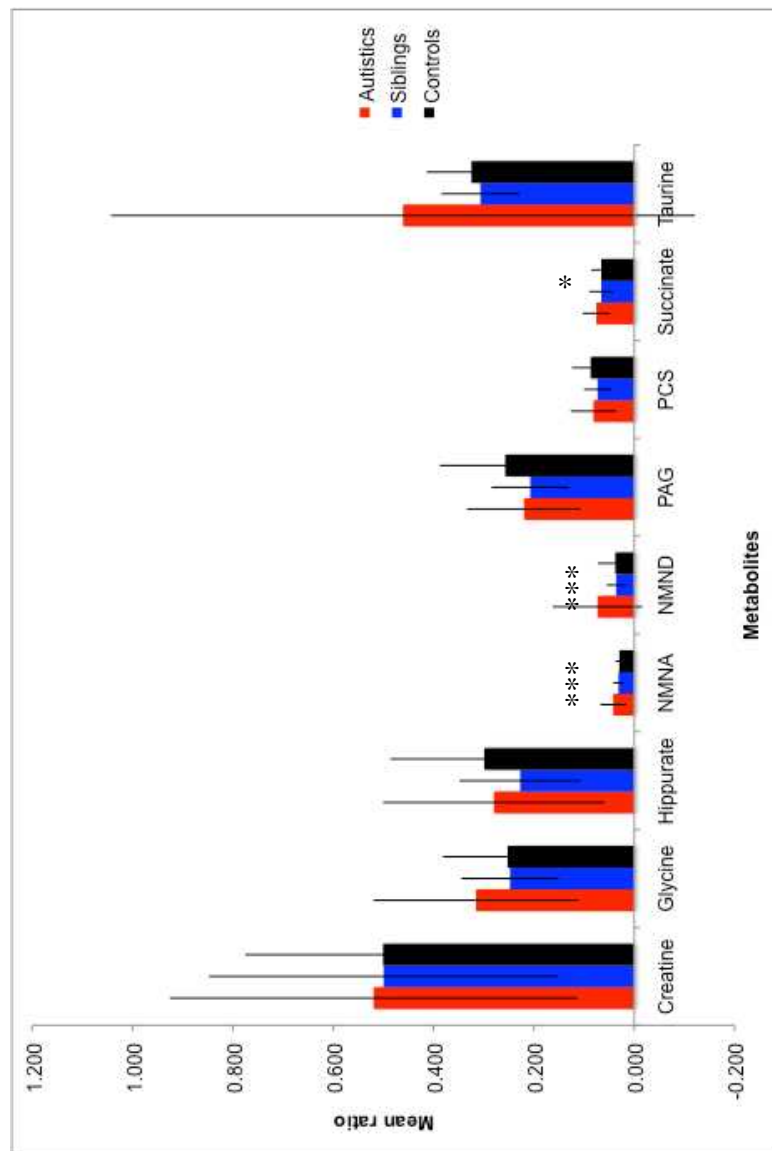
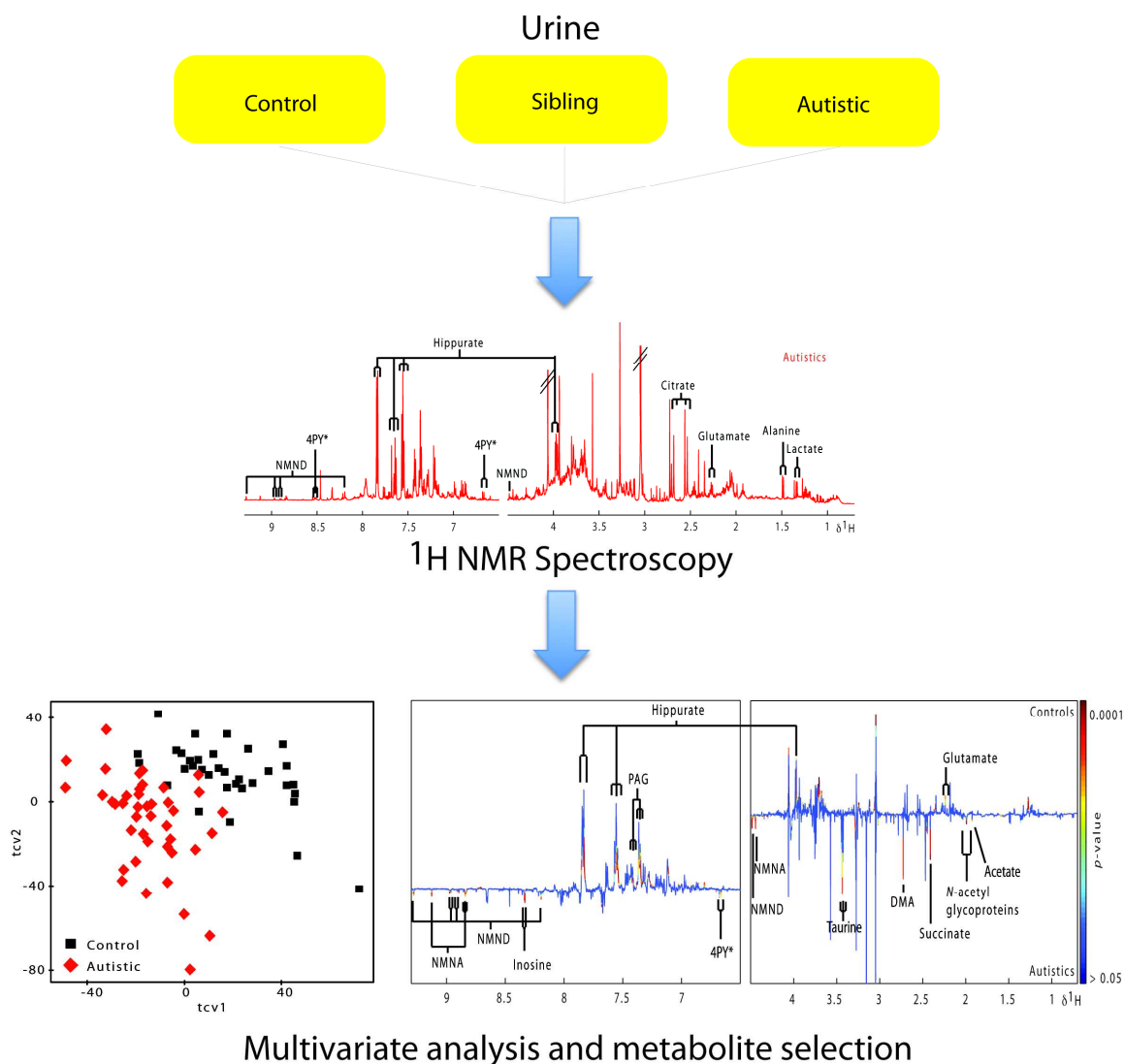


Figure 5



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.