Noninvasive urinary organic acids test to assess biochemical and nutritional individuality in autistic children

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ABSTRACT

Objectives: Quantitative organic acid testing can give information about potential problems, especially with energy production, neurotransmitter metabolism, intestinal dysbiosis and nutritional individuality which is very important in autistic children. The aim of this study was to find out potential differences between the levels of organic acids in the urine of autistic and non-autistic children.

Design and methods: The organic acids in the urine were determined by capillary gas chromatography/mass spectrometry (GC/MS). All overnight urine samples were collected from 35 autistic children and 36 neurologically normal children as healthy controls (4–10 years).

Results: Significant differences were found between the autistic children and the control group in organic acids: 2-oxoglutaric, isocitric, citric, 4-hydroxybenzoic, 4-hydroxyphenylacetic, hippuric, adipic, suberic (all with $p < 0.05$).

Conclusion: Organic acids test can be used to assess an individual need for nutrient and biochemical abnormalities, especially important for autistic children.

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Introduction

Autism is a complex metabolic disorder involving multiple organ systems, primarily the immunological, gastrointestinal and neurological systems. Many authors suggest that there exist multiple factors causing autism, among them nutrients, infections, genetic weaknesses and toxins [1–3]. Children with autism most commonly come under clinical attention between the second and third year of life. They are diagnosed on the basis of dysfunctions such as impaired social interaction, impaired communication and restricted and repetitive interests and activities. No child with core autism shows all of the characteristics, and some show only one or two symptoms in each key area. The American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders is the main diagnostic reference used by mental health professionals and insurance providers in the United States. The current (fourth) edition, which was published in 1994, is commonly referred to as the "DSM-IV." Autism spectrum disorders (ASD) called autism is characterized by Asperger Syndrome, Rett Syndrome, Childhood Disintegrative Disorder, and Pervasive Developmental Disorder Not Otherwise Specified (PDD-NOS). The DSM-IV category PDD-NOS is used to indicate a subgroup of ASD, where some, but not all, of the criteria for core autism are present. The term Asperger syndrome is often used to mark a subgroup at the more able end, in terms of social and communication handicaps, within the spectrum of ASD. Rett syndrome is a rare X-linked dominant progressive disorder that affects girls almost exclusively. In Childhood Disintegrative Disorder, children with normal development until the age of 2 years lose acquired skills such as language ability, bladder control and social development [4].

In the urine of many autistic children abnormal metabolites such as opioid peptides [5] and abnormal levels of different organic organics acids [6] were observed. A marked increase in analogs of Krebs cycle metabolites was found in the urine of two brothers with autistic features. The metabolites included citramalic, tartaric and 3-oxoglutaric acids [7]. Shaw [8,9] discussed microbial metabolites among American autistic children. The author suggests a strong connection between the presence of tartaric acid in the urine of autistic children and yeast. Moreover, higher values of oxalates are observed in the urine of autistic children than in that of non-autistic children. Oxalates and oxalic acid come from: the diet, from fungus (such as Aspergillus, Penicillum and Candida) and also from human metabolism. Probiotics may be very helpful in degrading oxalates in the intestine, and supplements of vitamin E, selenium and arginine reduce oxalate damage [8]. Indole acroyl glycine (IAG), a metabolite of IAA (indole acrylic acid) excreted in urine, was found in high concentrations in patients with autism [10]. Other authors observed the increased levels of eicosenoic acid and erucic acid in autistic subjects with developmental regression when compared to typically developing controls. The results suggest some metabolic or dietary abnormalities in the regressive form of autism [11]. Chugani and co-authors provided the evidence of altered energy metabolism in autistic children [12]. Relative carnitine deficiency in autistic patients

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is described in data [13]. The children with autism had significantly lower baseline plasma concentrations of methionine, homocysteine, cystathionine, cysteine and total glutathione and significantly higher concentrations of S-adenosylhomocysteine, adenosine and oxidized glutathione. This metabolic profile is consistent with the impaired capacity for methylation and increased oxidative stress in autistic children [14]. The levels of urinary creatinine in spot urine samples were analyzed for autistic children. A significant decrease in urinary creatinine concentration was found in the autistic group compared to the controls [15]. The metabolic abnormalities observed in autistic patients include alterations of TCA energy production, ammonia detoxification, reduced synthesis of omega-3 DHA, and abnormal cholesterol metabolism [16]. Poling and co-authors [17] suggest that the level of serum creatinine kinase was abnormally elevated in autistic patients. The data suggest that further metabolic evaluation is necessary in the case of autistic patients. Another paper [18] reports the investigation of the urinary excretion of amino acids in autistic children who did not receive any form of dietary intervention or treatment related to the digestive function. Significantly lower relative urinary levels of essential amino acids were revealed for autistic children.

Accumulations of organic acids in urine can indicate metabolic dysfunction, nutrient insufficiencies, or even microbial overgrowth [19]. The urinary organic acids test measures selected metabolites which serve as important diagnostic indicators of abnormal metabolism in the case of autistic children. The measurement of organic acids in urine evaluates four critical areas of metabolism: gastrointestinal function, cellular energy and mitochondrial metabolism, neurotransmitter metabolism, amino acid/organic acid balance as influenced by vitamin/mineral cofactors [20]. These areas of metabolism are very important in the case of autistic patients. Abnormal levels of organic acids can be traced to inherited or acquired enzyme deficiencies, build-up of toxicants, specific nutrient deficiencies, or drug effects [21]. The application of the organic acid testing to assess special nutrient requirements of individuals is a new diagnostic indicator and new idea in biomedical intervention in autism [22].

Coupled chromatographic techniques are the most effective methods of analyses of organic acids in biological fluids. Organic acids are mostly analysed by gas chromatography (GC) or GC–mass spectrometry (GC–MS). Applications using high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) were described in literature [23,24] with an advantage of shorter analysis time as compared to GC. However, due to the lower resolving power of HPLC and limitations in the detection of sensitivity of CE, GC methods are more suitable for the analysis of organic acids.

The main aim of the study was to find out whether there are differences between the levels of organic acids in urine of both autistic and non-autistic children.

Patients and methods

Patients and sample collection

The study was restricted to children with a diagnosis of autism in compliance with the criteria detailed in the *Diagnostic and Statistical Manual of Mental Disorders* (American Psychiatric Association, 1994) [25]. All overnight urine samples were collected from 35 autistic children (4–10 years) who underwent rehabilitation in the Clinic of Developmental of Dislocation Navicula-Centre in Lodz and 36 non-autistic children as healthy controls (4–10 years). All autistic children were assessed and diagnosed by clinicians specializing in the diagnosis and management of autistic children from the Navicula. In autistic children specific genetic disorders such as fragile X, Angelman syndrome, phenylketonuria and others were not observed. In the case of two children Asperger's syndrome was diagnosed. The autistic children were not on a gluten-free, casein-free diet. Eight children showed food intolerance to tomatoes, rye, eggs, yogurts. Urine samples were taken after usual dietetic limitation (tea, coffee, vanilla, chocolate, banana, walnut, grapefruit, camembert, and Coca-Cola). Other fruit and vegetables in the time of investigation were limited. None of the children received medications that could interfere with organic acids and their metabolites. All procedures were carried out, having the written consent of a parent. The study was approved by the Review Board of the Institute and performed in agreement with the Standards and Ethics in Biological Rhythm Research [26]. Urine was stored at −20 °C until analysis.

Sample preparation and analytical methods

Acids were extracted using ethyl acetate and diethyl ether and derivatized with BSTFA (N,O-bis[trimethylsilyl]trifluoroacetamide). The organic acids in the urine were determined by capillary gas chromatography/mass spectrometry (GC/MS). The trimethylsilyl (TMS) derivatives were separated using programmed temperature capillary gas chromatography on a HP-5MS column (Agilent 30 m × 0.25 mm i.d., film thickness 0.25 μm) in a Agilent 6890N gas chromatograph coupled to a Agilent 5970 mass selective detector. Compounds were identified from their electron impact (EI) mass spectra by comparison to spectra commercial mass spectral libraries (NIST, Wiley).

The oven temperature was programmed at 110 °C, increased to 200 °C at 9 °C/min, and then raised to 230 °C at 30 °C/ min. Helium was used as the carrier gas at a flow rate of 0.9 mL/min. The injection port, ion source, quadrupole and interface temperatures were 230 °C, 200 °C, 150 °C and 280 °C, respectively. The electron-impact (EI) mass spectra of the analytes were recorded by a total ion monitoring mode (scan range 40–550 m/z) to determine retention times and characteristic mass fragments. Standards of each compound were prepared at higher concentrations (1 mg/mL), derived and analyzed using full scan mass spectrometry (40–550 m/z) under the same chromatographic conditions [6]. The full mass spectra were needed to structurally identify the analytes and to obtain the retention times for each compound. Specific SIM groups were created in the mass spectrometer acquisition software for each analyte that included at least two representative ions from the mass spectra. Qualifying ions, monitored in the selected-ion-monitoring (SIM) mode were: m/z 73, 101, 143 for 2-oxoglutaric acid; m/z 73, 147, 273 for isocitric acid; m/z 211, 215, 273 for citric acid; m/z 193, 267 for 4-hydroxybenzoic acid; m/z 179, 296 for 4-hydroxyphenylacetic acid; m/z 105, 206, 308 for hippuric acid; m/z 111, 172, 275 for adipic acid; m/z 169, 187, 303 suberic acid and 147, 233 for malonic acid as internal standard (IS). The underlined ions were used for quantification.

The results are expressed as ratios to the urinary creatinine concentration in μmol/mmol creatinine. Creatinine was determined by the use of a high-performance liquid chromatography method reported elsewhere [27].

Statistical analysis

Data were statistically evaluated using statistical analysis package (StatSoft, Polska STATISTICA, version 9.0.). The Shapiro–Wilk test was used to check for normal distribution of the results. The Mann–Whitney U test was used to determine differences in single variables between values for other acids in the control and autistic group. The level of statistical significance was defined as $p < 0.05$.

Results

The method was applied to the analysis of urine samples collected from 35 autistic children (30 males and 5 females), and from 36 non-autistic children (28 males and 8 females). The level of 2-oxoglutaric acid, isocitric acid, citric acid, 4-hydroxybenzoic acid, 4-

hydroxyphenylacetic acid, hippuric acid, adipic acid, suberic acid is significantly higher ($p<0.05$; Mann–Whitney U test) in the urine of autistic children (4.69 ± 0.25 μmol/mmol creatinine, 20.10 ± 1.02 μmol/mmol creatinine, 218.67 ± 18.69 μmol/mmol creatinine, 1.49 ± 0.19 μmol/mmol creatinine, 13.32 ± 1.59 μmol/mmol creatinine, 225.66 ± 14.15 μmol/mmol creatinine, 14.92 ± 15.59 μmol/mmol creatinine and 7.72 ± 6.07 μmol/mmol creatinine, respectively) in comparison with that of the generally healthy children (3.72 ± 0.30 μmol/mmol creatinine for 2-oxoglutaric acid, 23.05 ± 1.47 μmol/mmol creatinine for isocitric acid, 170.34 ± 15.44 μmol/mmol creatinine for citric acid, 2.33 ± 0.23 μmol/mmol creatinine for 4-hydroxybenzoic acid, 6.27 ± 0.44 μmol/mmol creatinine for 4-hydroxyphenylacetic acid, 143.29 ± 12.10 μmol/mmol creatinine for hippuric acid, 2.13 ± 1.58 μmol/mmol creatinine for adipic acid and 1.71 ± 1.36 μmol/mmol creatinine for suberic acid). In detail results are shown in Table 1 and Figs. 1–3.

The autistic children did not receive any therapeutic treatment related to digestive function (such as antifungal medication, probiotics) and dietary intervention (gluten- and/or casein-free diet), nutritional supplements, or the hormone secretin. The levels of creatinine were 2.45 ± 2.25 μmol/mL for autistic children and 2.38 ± 2.12 μmol/mL for healthy children. In this group of autistic children the differences in levels of creatinine between healthy and autistic children were not observed. There were no significant differences between the levels of methylmalonic acid, ethylmalonic acid, methylsuccinic acid, fumaric acid, tartaric acid, methyladipic acid, stearic acid, azelaic acid, sebacic acid in the autistic and healthy children. Moreover, in the profile of organic acids of autistic children two compounds are significantly different in comparison with the control group. Arabinitol was nearly three times higher for the autistic group (134.21 ± 9.1 μmol/mmol creatinine) compared to the healthy children (47.96 ± 7.8 μmol/mmol creatinine). Concentrations of urinary tryptophan between the autistic

Fig. 1. Level of isocitric acid and citric acid in the urine of autistic and non-autistic children (μmol/mmol creatinine).

(5.22 ± 1.6 μmol/mmol creatinine) and healthy children (24.71 ± 5.3 μmol/mmol creatinine) were significantly lower.

Discussion

Human urine contains numerous metabolites and can provide the needed evidence for successful screening or chemical diagnosis of metabolic disorders and nutrient requirements. An increase in extraction can be non-specific because some metabolites are reported to be abnormally extracted in conditions not attributable to inborn errors metabolism (IEM) [28]. Abnormal extraction can be related not only to IEM, but to drug therapy, diet and physiological conditions.

The analysis of organic acids in human body fluids such as plasma, urine and cerebrospinal fluid is very important. This is necessary for the diagnosis of inborn errors of metabolism of amino and organic acids and for individual patient's diagnosis of nutrient requirements [19]. Obtaining cerebrospinal fluid and blood from children (especially healthy ones) is an ethical and methodological problem. These problems can be partly overcome through the use of urinary assays. Numerous studies have demonstrated the utility of urinary measurements in the case of autistic children [22,29–31]. The recent research has focused on a number of known neurometabolic disorders identified as having an autistic phenotype as well as on theories related to other metabolic abnormalities thought to contribute to the development of autism [32]. These metabolic disorders include: phenylketonuria (PKU), disorders of purine metabolism, biotinidase deficiency, disorders of cerebrospinal fluid (CSF) neurotransmitters such as deficiencies of folic acid, Smith–Lemli–Opitz syndrome (SLOS), methylmalonic acidemia, dicarboxylic aciduria and other organic acids acidemias [31–34]. Organic acids are important metabolites in major metabolic pathways such as the Krebs cycle or the pentose phosphate pathway. The accumulation of organic

Table 1
Descriptive statistics for levels of dicarboxylic acids in the urine of autistic and non-autistic children.

Organic acids	Mean (μmol/mmol creatinine)	Standard deviation	Min	Max	Significance
2-Oxoglutaric acid					
A	4.69	0.25	4.23	4.98	<0.05
C	3.72	0.30	3.23	3.98	
Isocitric acid					
A	20.10	1.02	16.98	21.45	<0.05
C	23.05	1.47	19.97	24.81	
Citric acid					
A	218.67	18.69	201.67	264.32	<0.05
C	170.34	15.44	134.98	190.21	
4-Hydroxybenzoic acid					
A	1.49	0.19	1.23	1.98	<0.05
C	2.33	0.23	2.10	2.87	
4-Hydroxyphenylacetic acid					
A	13.32	1.59	12.10	19.21	<0.05
C	6.27	0.44	5.49	7.02	
Hippuric acid					
A	225.66	14.15	202.12	273.11	<0.05
C	143.29	12.10	121.29	154.23	
Adipic acid					
A	14.92	15.59	2.36	57.33	<0.05
C	2.13	1.58	0.38	4.87	
Suberic acid					
A	7.72	6.07	0.60	22.68	<0.05
C	1.71	1.36	0.21	4.26	

A—autistic children; C—control group; Min—the lowest concentration of organic acids recorded in the group; Max—the highest concentration of organic acids recorded in the group.
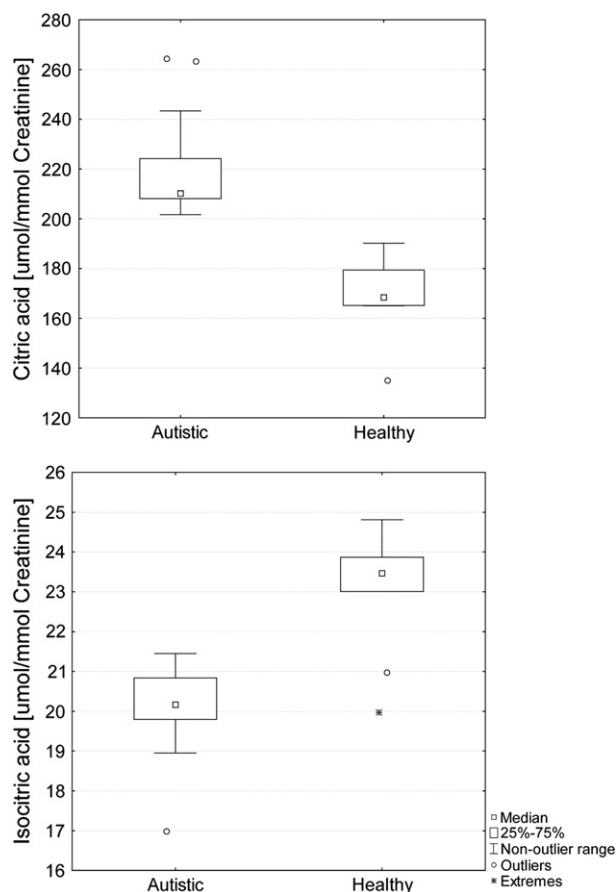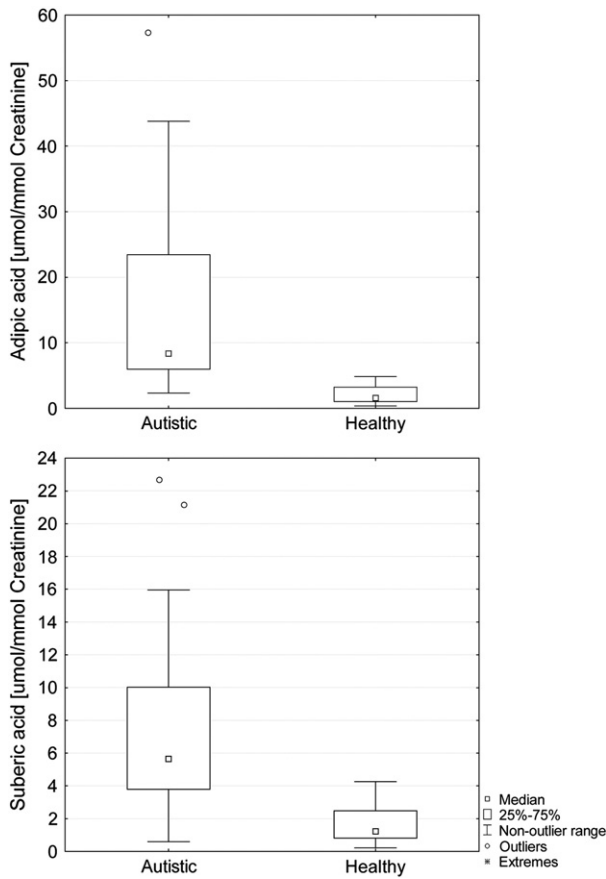
Fig. 2. Level of suberic acid and adipic acid in the urine of autistic and non-autistic children (μmol/mmol creatinine).

acids in biological fluids, especially in urine, can also provide useful information for an early diagnosis of metabolic disorders and neurological diseases [6,19,35]. The measurements of urine organic acids profile are used in modern nutritional medicine as a simple and sensitive test, which can demonstrate functional inadequacy of specific nutrients [20,21,30].

The quantitative organic acid profiling can assess: fatty acid metabolism, carbohydrate metabolism, energy production (cycle Krebs), B-complex sufficiency, methylation of co-factors, neurotransmitter metabolism, oxidative damage, detoxification status and intestinal dysbiosis due to bacteria and yeast [19,35]. Abnormal levels of organic acids can be used to meet an individual's need for a nutrient and can evaluate dysbiosis, which is especially important for children with autism. Shaw [7] tested urinary organic acid profiles in two autistic brothers. These tests revealed a consistent excretion of a number of compounds of possible microbial origin identified as the carbohydrate arabinose, analogs of normal Krebs cycle intermediates including 3-oxoglutaric acid, tartaric (3-hydroxymalic) acid, citramalic (methylmalic) acid, and a new tentatively identified analog of citric acid (carboxycitric acid) not previously reported in the medical literature. The hydroxymethylfurancarboxylic acid, furandicarboxylic acid, furancarbonylglycine, and 3-(3-hydroxyphenyl)-3-hydroxypropionic acid were found in urine as chemicals of possible microbial origin.

An increased level of citric acid observed in the presented investigations can suggest an amino acid deficiency or problems with protein metabolism in autistic children. Succinic acid cannot play its role in the production of cellular energy via the citric acid cycle when coenzyme Q10 (CoQ10) is inadequate. Elevated succinic acid
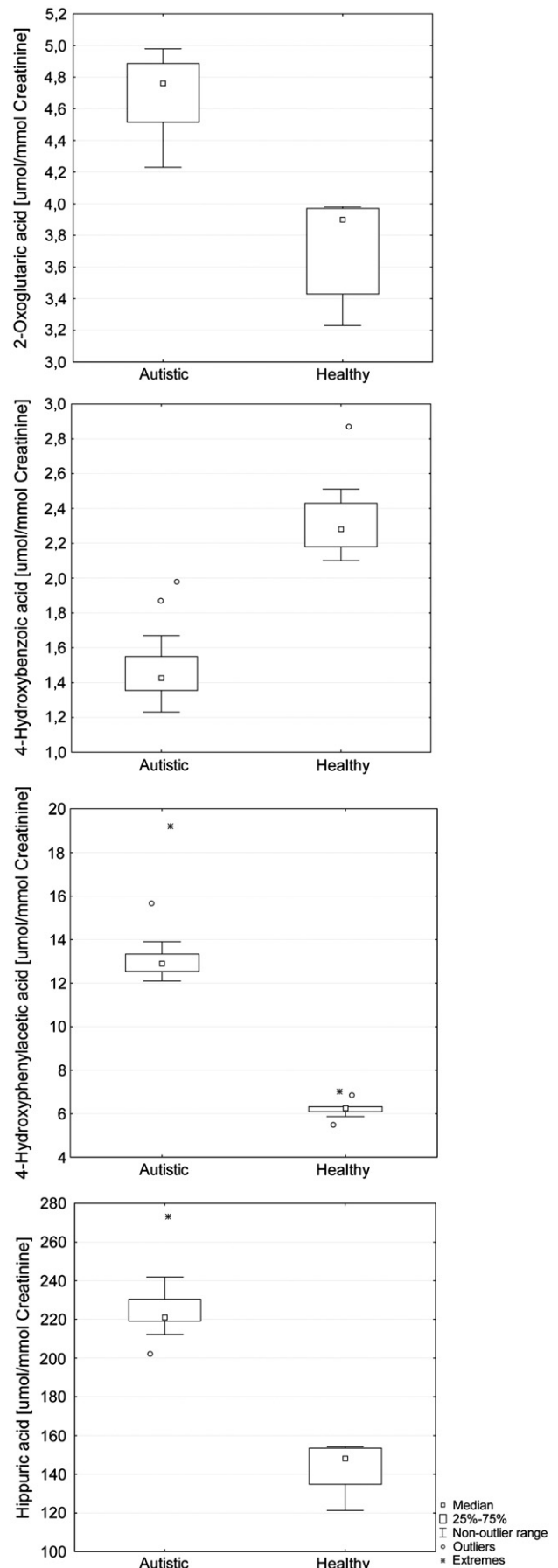

Fig. 3. Level of 2-oxoglutaric, 4-hydroxybenzoic, 4-hydroxyphenylacetic, hippuric acid in the urine of autistic and non-autistic children (μmol/mmol creatinine).

excretion is a marker for deficiency of CoQ10 and riboflavin in children with autism. Adipate and suberate are the products of incomplete oxidation in the omega-oxidation pathway. Increased urinary extraction of adipic and suberic acids is well known as a marker of fatty acid peroxidation in diabetes [36]. Supplementation of carnitine and riboflavin is indicated when adipic and suberic acids are elevated [19,31,37].

The 4-hydroxyphenylacetic levels were significantly higher in autistic children and this may reflect increased gut metabolism of tyrosine secondary to bacterial overgrowth. Hippuric acid levels are also influenced by gut bacterial amino acid metabolism but, also and more importantly, by ingestion of benzoic acid containing foodstuffs. Urinary 2-oxoglutaric acid may be influenced by many factors including urinary tract bacterial activity [38].

Tryptophan is a precursor to the neurotransmitter serotonin, which is strongly linked to autism. Low urinary tryptophan has previously been reported as anomalous for autistic children [18]. Lower levels of tryptophan may lead to the worsening of autistic symptoms such as mild depression and increased irritability [18,30].

D-arabinitol is a metabolite of most pathogenic Candida species and urinary extraction is elevated in autistic patients [9]. The use of probiotics can be effective in reducing the level of D-arabinitol in the urine of children with autism.

In the literature pertaining to autism research there is information about the gut–brain connection and the strong association of various factors influencing digestive function and nutritional uptake in autism. There are treatment strategies consisting in a correction of biochemical imbalance in autistic patients such as: eradication of fungal (Candida) overgrowth and restoration of gut integrity, removal of potential sources of opioid peptides from the diet (gluten-free/casein-free diets) [18,39]. Chronic biochemical imbalance can be a primary factor in the development of many complex diseases such as autism. Researchers [14] report that the autistic children have a severely abnormal metabolic profile, indicating increased vulnerability to oxidative stress.

The findings from of this study indicate that the abnormalities in urinary organic acid excretion between autistic and healthy children are present. These results do not provide strong evidence for the theory that abnormal metabolites play a role in the pathogenesis of autism spectrum disorders although they suggest some metabolic or dietary abnormalities in the autism.

The metabolome analysis can be effective for detecting abnormal metabolic and/or nutritional conditions in autistic children, including acquired vitamin deficiencies and the potential biological response to drugs or excess nutrient loading.

This simple, sensitive and noninvasive test can reveal evidence of functional inadequacy of specific nutrients and biochemical disorders in autistic children.

A future study would concern the treatment strategies which will enable biochemical balance in autistic children. The therapeutic treatments are related to digestive function and dietary intervention.

Acknowledgments

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