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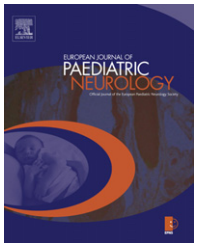

Original article

A potential pathogenic role of oxalate in autism

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ABSTRACT

Background: Although autistic spectrum disorders (ASD) are a strongly genetic condition certain metabolic disturbances may contribute to clinical features. Metabolism of oxalate in children with ASD has not yet been studied.

Aim: The objective was to determine oxalate levels in plasma and urine in autistic children in relation to other urinary parameters.

Method: In this cross-sectional study, plasma oxalate (using enzymatic method with oxalate oxidase) and spontaneous urinary calcium oxalate (CaOx) crystallization (based on the Bonn-Risk-Index, BRI) were determined in 36 children and adolescents with ASD (26 boys, 10 girls) aged 2–18 years and compared with 60 healthy non-autistic children matched by age, gender and anthropometric traits.

Results: Children with ASD demonstrated 3-fold greater plasma oxalate levels [5.60 (5th–95th percentile: 3.47–7.51)] compared with reference [(1.84 (5th–95th percentile: 0.50–4.70) μmol/L ($p < 0.05$)] and 2.5-fold greater urinary oxalate concentrations ($p < 0.05$). No differences between the two groups were found in urinary pH, citraturia, calciuria or adjusted CaOx crystallization rates based on BRI. Despite significant hyperoxaluria no evidence of kidney stone disease or lithogenic risk was observed in these individuals.

Conclusions: Hyperoxalemia and hyperoxaluria may be involved in the pathogenesis of ASD in children. Whether this is a result of impaired renal excretion or an extensive intestinal absorption, or both, or whether Ox may cross the blood brain barrier and disturb CNS function in the autistic children remains unclear. This appears to be the first report of plasma and urinary oxalate in childhood autism.

Abbreviations: ASD, Autistic spectrum disorders; Ox, oxalate; CaOx, calcium oxalate; BRI, Bonn-Risk-Index.

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1. Introduction

The autism spectrum disorders (ASD), including classical autism, are regarded as a group of complex developmental disorders associated with life-long disability, of which prevalence during growth is considerably greater than previously thought.[1] This may reflect an increasing incidence of this condition.[2,3] Despite decades of research and high level of evidence, the etiology of ASD remains unclear, and biological causes are poorly understood.[4,6]

Research has emphasized that ASD is strongly a genetic disorder.[1,4,7–9] A wide range of abnormalities in central nervous system has been reported in autistic patients, including changes in brain size and reduced neurons in certain specific brain regions,[6,10–13] and at least some of these features may be due to earlier deterioration of brain formation.[13] Various theoretical approaches to autism have been discussed. Independent of the genetic background, a number of additional pathways, interactions between genetic and environmental factors and also co-morbidities have been reported in autism, including advanced maternal age and parity,[14] environmental contribution to the condition, altered neurochemistry (in particular high peripheral serotonin levels), immunoexcitotoxic mechanisms, altered oxidative–reductive capacity, disturbed sulfur chemistry and behavioral symptoms, food allergies, intestinal dysbiosis, recurrent infections and possible altered immune response.[1,15–25] Among others, a high prevalence of gastrointestinal symptoms is frequently reported in ASD children which may be alleviated using dietary intervention or elimination diet.[17] Some hypotheses appear regarding the pathogenic role of nutrients or trace elements in ASD, however, the level of evidence is not sufficient. The alterations in nutritional metabolism in the development of childhood ASD have been widely studied but the results are conflicting.

Metabolism of oxalate in children with autism has not been confirmed by laboratory tests. Thus, we hypothesized that oxalate may contribute to, or at least play a role in neuropsychiatric damage and behavioral dysfunction in ASD. The objective of this study was to determine oxalate levels in plasma and urine in children with autism in relation to other urinary parameters (calciuria, citraturia) and spontaneous urinary calcium oxalate crystallization.

2. Methods

2.1. Study participants

The study was conducted in 36 Caucasian children and adolescents with autism (26 boys, 10 girls) aged 2–18 years (median 5.6 yrs; 5th percentile − 2.4 and 95th percentile − 14.6). These patients were recruited from different specialized centers, including our teaching hospital, and were followed in the departments and clinics (developmental neuropsychology, psychiatry, gastroenterology, metabolic, pediatric nephrology) of the University Children's Hospital in Bialystok (Poland). They presented a variety of clinical features, however, the core elements, including abnormal cognitive development, impairment in social interactions, stereotyped or unusual behaviors, deviance in communication, were typical for autistic spectrum disorders. None of these children had history of seizures or epilepsy. The diagnosis of autism was ascertained using current ICD-10 criteria (F 84.)[26] and DSM-IV (Diagnostic and Statistical Manual of Mental Disorders IV) guidelines, and was confirmed by independent board-certified psychiatrists in different centers including ours.

The reference group consisted of 60 healthy children (30 boys and 30 girls) matched by age (median 5.4 years; 5th percentile − 2.9 and 95th − 15.1) and anthropometric traits (Table 1). Anthropometric measurements (weight, height) were performed using electronic scale (Seca, Germany) and Martin anthropometer, and Body Mass Index (BMI) was calculated using standard formula.

All children met the criteria of the age-related standard energy and dietary intakes recommended in Poland.[27] No dietary restrictions (e.g. milk-free, vegan or gluten-free diets) were reported in the autistic children. There were no diseases known to affect calcium and phosphate metabolism, no endocrine co-morbidity or antibiotic treatment before tests. None of the children were diagnosed with celiac disease or inflammatory bowel diseases. None of them had a family history of urolithiasis (first-degree relatives). All study subjects were screened for urolithiasis using high resolution renal ultrasonography (Toshiba SSH-140A apparatus; probe Convex 3.75 MHz, operated by one trained person) and none of them had urinary stones. None had urinary tract stenosis or urinary tract infection. Urinary dipstick testing (Bayer Diagnostics Mfg. Ltd, Bridgend, UK) detecting nine parameters, including leukocytes and protein, did not show abnormalities. None of the study participants had cystinuria (based on the negative result of sodium nitroprusside test) or hyperuricosuria (24-h urinary uric acid excretion unrevealing). The study protocol was approved by the Ethical Committee of the Medical University of Bialystok.

3. Methods

Oxalate levels were determined in blood plasma samples after a night break without taking food (10–12 h) using enzymatic method with oxalate oxidase derived from 10-days old barley seedling adding oxalate to stabilize the endogenous plasma oxalate.[28] This method has been previously validated in children and has provided a comprehensive reference database.[29]

In this study, spontaneous urinary calcium oxalate (CaOx) crystallization was assessed with the Bonn-Risk-Index (BRI) using the method by Laube and colleagues.[30] Each studied child had a 24-h urine collection into sterile containers, without additional preserving substances, which was stored at temperature of 4 °C. The testing was always performed twice using the same urine collection from each subject. Two consecutive urine samples (each 100 ml) were incubated immediately after collection, at a temperature of 37 °C and the calcium ion [Ca^{2+}] concentration was measured using calcium ion-selective electrodes of type Rapilab 855 (Bayer, Germany) and titrated with ammonium oxalate solution (40 mmol/L) at a rate of 0.75 mL per minute. The onset of spontaneous

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crystallization was detected using an Eppendorff photometer (filter 585 nm) with a decrease in light transmission to 98% of the initial value. Mean value was derived from the amount of added ammonium oxalate (Ox^{2-}) and calculated for 200 ml of urine.[31] Each analysis was repeated twice. The results of BRI were presented as [Ca^{2+}] mmol/L/(Ox^{2-}) mmol = 1/L. Calibration and quality assurance procedure, based on the calibration curves, were made appropriately. The quality assessment of the method was based on creatinine loss from the 24-h urine sample. Urine collections in which creatinine levels were below the 10th centile, relative to age, were rejected. Prior to measurement of ionized Ca in the urine, pH was determined in each urine sample using microcomputer pH-Meter CP-315M (Elmetron). Urine calcium, and creatinine concentrations were assessed with the Cobas-Integra 800 analyzer and Roche reagents. Urine oxalates were examined in a standard way (Trinity Biotach) and citrates were examined using a commercial set (Boehringer Mannheim/R-Biopharm). All 24-h urine samples were collected from inpatients, with parental assistance, on the second or third day of the hospitalization.

Statistical analysis was performed using the Statistica 8.0 PL. Liliefors, Kolmogorov–Smirnov and W Shapiro–Wilk tests were done in order to determine the distribution modality of the data. The differences between autistic and healthy children were determined with Mann–Whitney test used for the analyses of two non-parametric independent variables. Further, assessment of the rank of two independent variables was conducted using Spearman correlation and considered statistically significant at $p < 0.05$. For the purpose of plotting the curve of spontaneous crystallization (i.e. an association between the number of calcium ions and the amount of added ammonium oxalate leading to the spontaneous crystallization), we used the computer program: the range of scattering with the special option of adding curves.

4. Results

The plasma oxalate levels were found to be 3-fold greater in the autistic children [5.60 (5th–95th percentile: 3.47–7.51)] compared with reference [1.84 (5th–95th percentile: 0.50–4.70) μmol/L ($p < 0.05$)]. Our results showed that children with autism demonstrated over 2.5-fold greater urinary oxalate levels compared with healthy peers: 1.07 (5th–95th percentile: 0.48–2.14) mmol/1.73m^2/24 h vs. 0.41 (5th–95th percentile: 0.11–0.46) mmol/1.73m^2/24 h ($p < 0.05$). Patients with autism had also a significantly lower urinary [Ca^{2+}] levels

Table 1 – The characteristics of children with autism compared with healthy reference, including anthropometry, urine and plasma parameters.

	Patients with autism $n = 36$	Healthy controls $n = 60$
Age (years)	5.6 (2.41–14.66)	5.35 (2.91–15.08)
Height (cm)	111.5 (93.00–175.00)	111.75 (95.00–178.00)
Weight (kg)	20.25 (13.50–59.90)	19.50 (13.80–63.00)
Body Mass Index (kg/m^2)	15.77 (13.54–20.42)	15.53 (12.53–23.43)
Urine		
Urine volume (ml/kg/24 h)	37.35 (15.43–60.62)	42.85 (9.41–66.66)
pH of urine	6.60 (6.20–7.40)	6.46 (5.70–7.50)
Oxalate (mmol/1.73m^2/24 h)	1.07 (0.48–2.14)*	0.41 (0.11–0.46)
Calciuria (mg/kg/24 h)	1.67 (0.71–4.59)	1.55 (0.53–3.96)
Citrate in urine (mg/g creatinine/24 h)	673.45 (187.55–952.98)	585.68 (427.14–1615.51)
[Ca^{2+}] mmol/L	0.18 (0.10–0.60)*	0.23 (0.12–0.88)
(Ox^{2-}) mmol	2.57 (0.46–3.12)	2.10 (0.37–10.12)
BRI 1/L	0.06 (0.03–1.47)	0.12 (0.02–1.79)
BRI/kg (1/L × kg)	0.004 (0.002–0.085)	0.006 (0.001–0.076)
BRI/1.73m^2 (1/L × m^2)	0.17 (0.08–3.75)	0.26 (0.03–3.49)
BRI/BMI (m2/L × kg)	0.004 (0.002–0.10)	0.007 (0.001–0.10)
BRI/g creatinine (1/L × g)	0.23 (0.11–3.56)	0.34 (0.04–3.65)
The equation for hyperbola		
Median	[Ca^{2+}] = 0.3148/(Ox^{2-})	[Ca^{2+}] = 0,5232/(Ox^{2-})
5th percentile	[Ca^{2+}] = 0.1224/(Ox^{2-})	[Ca^{2+}] = 0,2128/(Ox^{2-})
95th percentile	[Ca^{2+}] = 0.5529/(Ox^{2-})	[Ca^{2+}] = 1,5479/(Ox^{2-})
Plasma		
Ox (μmol/L)	5.60 (3.47–7.51)*	1.84 (0.50–4.70)
Ox (mg/dL)	0.05 (0.03–0.06)*	0.016 (0.004–0.042)
Ox/1.73m^2 (μmol/L × m^2)	11.15 (4.84–17.93)*	3.73 (0.91–10.22)
Ox/1.73m^2 (mg/dL × m^2)	0.10 (0.04–0.16)*	0.03 (0.008–0.092)
Ox/kg (μmol/L × kg)	0.26 (0.08–0.45)*	0.08 (0.02–0.24)
Ox/kg (mg/dL × kg)	0.002 (0.0007–0.004)*	0.0008 (0.0002–0.002)
Ox/Cr (mg/mg)	0.13 (0.04–0.17)*	0.036 (0.009–0.106)

*($p < 0.05$) – U Mann–Whitney test (significant differences in urine parameters between autistic and healthy children). Values are shown as median and the range (5th–95th percentiles).
Ox – denotes oxalate.

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relative to the healthy reference: 0.18 (5th–95th 0.10–0.60) mmol/L vs. 0.23 (5th–95th percentile: 0.12–0.88) mmol/L. Other traits such as urinary volume, urine pH, citraturia, calciuria or adjusted CaOx crystallization rates based on the BRI, did not differ between the two groups (Table 1).

Furthermore, urinary CaOx concentrations correlated with age, height, weight ($R = 0.47$; 0.53; 0.45 respectively; all $p < 0.05$) and also correlated positively with plasma oxalate levels, when adjusted for 1.73 m^2 body surface ($R = 0.68$), body weight ($R = 0.62$) and normalized per gram creatinine ($R = 0.69$) (all $p < 0.05$). Fig. 1 shows relationships between plasma oxalate levels and oxalate excretion in the urine: similar trends are present in both autistic and healthy children, suggesting that increased plasma oxalate levels are associated with hyperoxaluria.

The differences in plasma oxalate levels between autistic and healthy individuals and the rates of spontaneous urinary calcium oxalate crystallization, based on the relationship between [Ca^{2+}] (mmol/L) and the amount of added (Ox^{2-}) (mmol), are shown in Fig. 2, where black squares denote children with autism and blue ones denote healthy subjects.

5. Discussion

The complex and multifactorial etiology of early neurodevelopmental damage in ASD is an essential issue in the consideration of the disease and, so far, there is no consensus about the neurological pathophysiology of ASD.[32] Multiple combinations of genes are now being proposed to lead to the underlying mechanisms of autistic phenotype, and these combinations of genes may contribute to metabolic disorders found in children with ASD and be responsible for clinical symptoms.[33,34] Nevertheless, based on existing findings, the causal pathways in autism are still difficult to explain. Several metabolic and biochemical disorders have been reported in children with autism, including increased urinary concentration of certain peptides and water soluble components,[35] lower urinary amino acids excretion,[36] increased coproporphyrin levels,[37] excessive protein catabolism,[38] lipid peroxidation biomarkers suggesting increased oxidative stress,[39] lower tryptophan level and serotonergic disturbances, as well as insufficient melatonin production.[40,41]

As there is no published data regarding oxalate homeostasis and ASD, it is worthwhile evaluating these associations. In this study, children with ASD had an increased plasma oxalate levels (approximately 3-fold greater relative to healthy population) and also demonstrated a proportionally increased urinary oxalate excretion. Interestingly, these children did neither have kidney stone disease nor even a tendency to form calcium oxalate crystals in the urinary tract.

In the human body, oxalate is the final product of the degradation process of some amino acids and ascorbate.[42–44] The homeostasis of oxalate is a derivative of the absorption and transportation in the digestive system and both renal and intestinal excretion.[24,45] It is well documented that urinary oxalate is one of the major promoters of calcium oxalate stone formation in adults and children.[46,47]

Assuming the amount of oxalate in urine (as a strong crystallization promoter) is elevated, the individuals with ASD should be at risk of kidney stone disease. However, the BRI values reflecting spontaneous crystallization were paradoxically normal or even lower in these children (median (0.06 (5th–95th percentile 0.03–1.47) 1/L)) compared to studied controls or to healthy reference (median 0.26 (5th–95th percentile 0.06–1.93) 1/L).[48] This may have been partly due to a relatively low calciuria found in autistic children, as urinary calcium is thought another important crystallization promoter. The ASD children had also normal citraturia rates (median 673.45 (5th–95th percentile 187.6–953) (mg/g creatinine/24 h)) presumably preventing crystal formation. The link between autism and kidney stone disease has not been reported, and, similarly in the light of our findings, there was no lithogenic risk in individuals with ASD despite hyperoxaluria. One cannot exclude the possibility of an alternative profile of oxalate metabolism which may occur in children with autism. Whether there are, for example, oxalate crystals/deposits in other tissues, including brain, is not known.

It remains to be determined what may be causing hyperoxalemia and hyperoxaluria in children with autism considering that the renal function is normal in these individuals. Excessive permeability of the gut in autism was described by d'Eufemia in 1996, and that could lead to the condition called "enteric hyperoxaluria". Gastrointestinal disorders are common in children with ASD,[15,18] so there is a possibility that chronic or subclinical intestinal inflammation, with ileocolonic lymphoid tissue hyperplasia, may be responsible for an increased absorption and availability of oxalate,[15,49] and that may more seriously affect children with autism who were excluded from this study. Although autistic children in this study did not demonstrate apparent clinical malabsorption or maldigestion, an imbalance of intestinal microflora may have been involved in the altered metabolism of oxalate. Gut dysbiosis which is frequently reported in ASD may be associated with absence of certain bacterial strains participating in oxalate degradation in the colon (e.g. Oxalobacter formigenes) which may have been killed back by exposures to antibiotics. Patients with a history of antibiotic use were excluded from this study.

Overproduction of oxalate taking place in liver should also be taken into consideration. Problems in the B6 chemistry[50] which have been described in autism could impair the handling of oxalate by compromising the activity of the enzyme AGT, the enzyme which causes primary hyperoxaluria type 1. Finally, the activity of transporters in the kidney may be impaired. These transporters are responsible for transporting oxalate out of the blood and into kidney tubule cells on the basolateral side, and are paired in activity with other transporters which then secrete that oxalate from the apical side into the urine. There is a new interest in the transporter SLC26A6 (also called PAT1 or CFEX) which functions in both the intestine and the kidney. Studies conducted in patients with primary hyperoxaluria type 1 and 2 have identified different SLC26A6 variants.[51] Some reports suggest that mutations of this anion transporter which is responsible for mediation of chloride/oxalate exchange may cause or modify hyperoxaluria in humans.[51–54]

Oxalate and sulfate also share regulation in transport via sulfate/oxalate exchange. For this reason, potential

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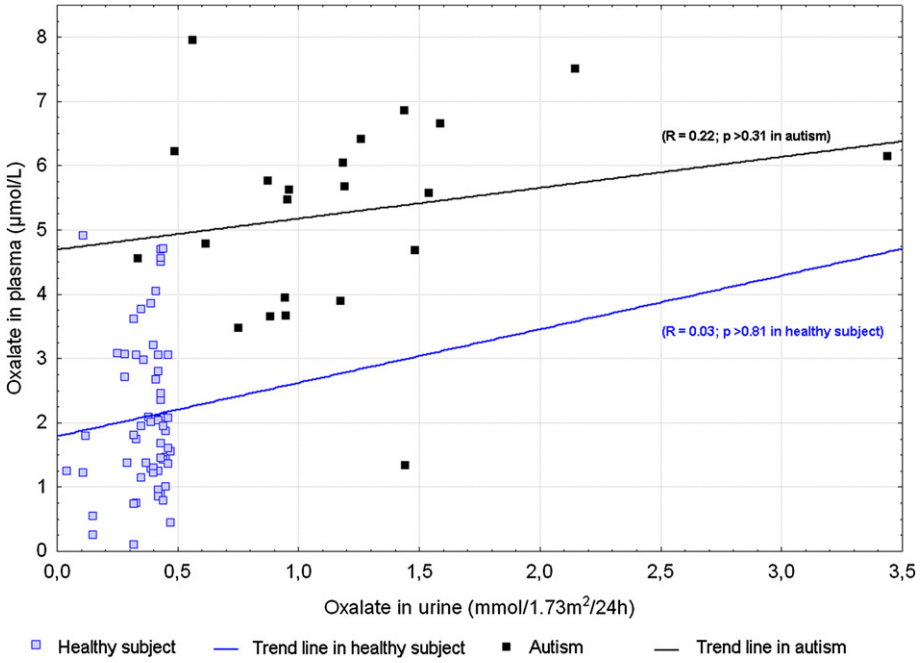

Fig. 1 – Urinary oxalate excretion in relation to plasma oxalate levels in children with autism and healthy reference. Trend lines are shown for the two groups.

movement of oxalate across membranes must be seen in context with the problems in sulfate chemistry that have been found in autism and were mentioned earlier. A mouse developed with no activity for the sulfate transporter called NaS1 has low sulfate in plasma and high sulfate in urine similarly to Waring and Klovrza's finding in autism.[22] Another mouse was developed lacking the sulfate/oxalate exchanger called SAT-1. Other exchangers that transport

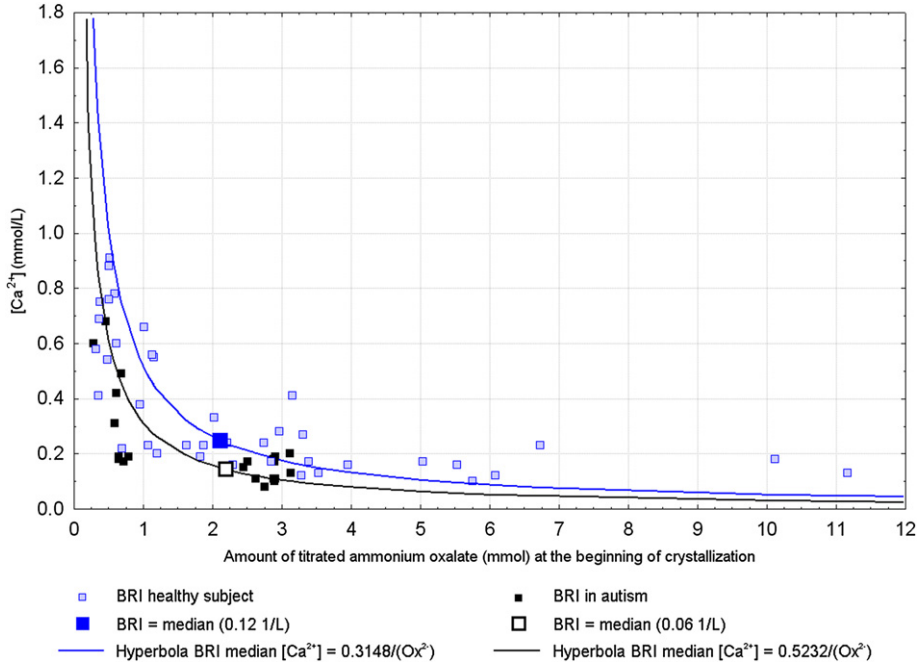

Fig. 2 – The spontaneous crystallization of calcium oxalate (CaOx) in the urine of healthy children (blue squares) compared with those with autistic spectrum disorders (black squares). X-axis – amount of ammonium oxalate (Ox^{2-}) (expressed in mmol) necessary for the onset of spontaneous crystallization. Y-axis – concentration of calcium ions ($[Ca^{2+}]$) before the addition of Ox^{2-}. The large blue square – median Bonn-Risk-Index (BRI) for healthy children, the large white square – median BRI for children with ASD. The blue hyperbola crossing through the large blue square defines median values in healthy children, black hyperbola crossing the large white square corresponds to median values in children with ASD. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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both oxalate and sulfate exist in erythrocytes and in the placenta. An understanding of how these transporters interrelate with oxalate trafficking may help determine the extent to which the sulfation issues in autism could modify the trafficking of oxalate in the kidney, the intestine, and even in the brain.

Oxalate levels in children with autism who had no obvious gastrointestinal problems were considerably lower than those reported in patients with severe primary hyperoxaluria, however the data may be different in the children with autism who do have gastrointestinal disease. However, neither oxalate transport in human brain nor, in particular, oxalate effect on the brain in autistic disorders have been investigated so far. Only few postmortem studies have reported cases of acute brain damage with the presence of oxalosis caused by ethylene glycol poisoning,[55–57] but severe or chronic neurotoxicity was reported as a side effect of the cancer drug oxaliplatin, with the toxicity determined to come from the oxalate metabolite. Encephalopathy has also been described after ingestion of certain high oxalate foods. It cannot be excluded that slightly increased plasma oxalate levels and small amounts of oxalate depositions may interact with central nervous system in children with ASD. Thus, phenotypic analysis of SLC26A6 variations or detection of oxalate transport mechanisms in brains of individuals with autism could lead to our further understanding of these associations.

Our observation does not suggest that oxalate is an essential indicator of metabolic disorder in autism, and our selection criteria may have under represented the range of plasma and urinary oxalate that would occur in a full range of autistic patients that included those with special diets, with seizures, with histories of antibiotic use or with serious gastrointestinal disease. Our relatively small number of studied children may obscure true relationships and limit inferences that could be made. We are aware that the reason for an increased oxalate level or a role for oxalate in neurodevelopmental damage still remains unclear. Nevertheless, the coincidence of hyperoxalemia and autism with absence of urolithiasis suggests a relevant association, particularly in the context of future dietary recommendations and treatment perspectives for children with ASD.

6. Conclusions

In summary, hyperoxalemia and hyperoxaluria may be involved in the pathology of autistic spectrum disorders in children, although data is insufficient to determine its relevance, if at all, to pathogenesis. Some treatment options such as low oxalate diets, probiotic treatment (e.g. with *Oxalobacter formigenes*), supplementation with recombinant enzymes, modification of intestinal oxalate secretion or perhaps oxalate binding treatments may be helpful in these children. Whether improvement of oxalate status will alleviate behavioral changes, and cognitive and social functions is currently under investigation.

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The two first authors (JK and TP) contributed equally to this work.

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