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ScienceDirect
Journal of Nutritional Biochemistry 70 (2019) 38–46
Journal of Nutritional Biochemistry

The concomitant lower concentrations of vitamins B6, B9 and B12 may cause methylation deficiency in autistic children

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Received 9 May 2018; received in revised form 20 February 2019; accepted 17 April 2019

Abstract

Autism spectrum disorder (ASD) is characterized by severe and persistent difficulties in social communication and social interaction at multiple levels. Recently, metabolic disorders have been associated with most cases of patients with ASD. The aim of this study was to investigate, through a new and more sophisticated mass technique, such as UHPLC-mass spectrometry (Q-exactive analyzer), alteration in metabolisms analyzing ASD children urine samples from children showing simultaneous vitamin B6, B9 and B12 deficiencies. This in order to study how these concurrent deficiencies may influence some phenotypic aspects of autistic disorder. Thus, urinary metabolic patterns specific to ASD were explored at an early age in 60 children with ASD, showing lower three vitamins levels, and 60 corresponding controls (age group 3–8, M: F=42:18). The results showed significant block of cystathionine formation with consequent accumulation of homocysteine. A lower glutathione levels (GSH), with reduction of essential intracellular reducing environment required for normal immune function, detoxification capacity and redox-sensitive enzyme activity. Increased concentration of 5-methyltetrahydrofolate, which leads to a lower availability of methyl group and significant decrease in urinary methionine and S-adenosyl-L-methionine (SAM) concentrations, the major methyl donor. The latter justify the well-known reduction in protein and DNA methylation reported in autistic children. As a final consideration, the concomitant deficiencies of all three B vitamins, recorded in a significant number of autistic children, suggests that intestinal dysbiosis in these patients may be the main cause of a reduction in their absorption, in addition to the genetic mutation of a specific gene.
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Keywords: Autism; Metabolomics; Vitamin B6; Vitamin B12; Vitamin B9; Methylation

1. Introduction

Among all autism spectrum diseases, autism is doubtless the most severe and can be distinguished from other neurodevelopmental conditions such as Asperger syndrome (AS) and the non-otherwise specified pervasive developmental disorder (PDD-NOS) [1], which shows specific peculiarities. Autism spectrum disorders (ASD) are increasingly recognized as a public health problem, which have increased in the last two decades from 2–5/10,000 to 1/68 children [2,3]. Improvements in diagnostic criteria have contributed to this evident increase [4]. Several studies have shown the presence of alterations in neuropsychological function and of learning disabilities in subjects affected by metabolic alterations, which showed the presence of a progressive neuropsychological decline in areas of intellect, attention, memory and executive functions [5]. Recently, some metabolic abnormalities have been associated with various diseases involving behavioral disorders associated with autism. In fact, although autism spectrum disorder (ASD) is still diagnosed through behavioral observation due to a lack of laboratory biomarkers, recent metabolomics investigations on human biofluids such as urine and plasma [6,7] provided a sensitive tool to identify metabolite profiles which could greatly aid clinicians in providing earlier and more reliable diagnoses.

A comparative analysis of the urine and plasma between ASD and control individuals showed that some metabolic pathways, most distinctive of young Italian autistic children, largely overlap with those found in rodent models of ASD, suggesting that autistic patients may share some metabolic abnormalities. Interestingly, changes in DNA methylation at differentially methylated CpG sites also correlated with total childhood autism symptom test scores [8,9].

Most of the urinary metabolites displaying the largest differences between young ASD and control children belonged to the tryptophan and purine metabolic pathways [10]. In ASD children, tryptophan is preferentially transmuted to xanthurenic acid and quinolinic acid (two catabolites of the kynurenine pathway) at the expense of kynurenic acid and especially of melatonin. In addition, the gut

Abbreviation: ASD, autism spectrum disorder; CSB, cystathionine β-synthase; CSE, cystathionine γ-lyase; GSH, glutathione; GSSG, oxidized glutathione disulphide; MS, methionine synthase; SAH, S-adenosyl-L-homocysteine; SAHH, SAH hydrolase; SAM, S-adenosyl-L-methionine; MTase, methyltransferase; MMA, methylmalonic acid; P5P, pyridoxal 5′-phosphate; THF, tetrahydrofolate; UHPLC, ultra-high-performance liquid chromatography; 5-methylTHF, 5-methyltetrahydrofolate

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https://doi.org/10.1016/j.jnutbio.2019.04.004
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microbiome contributes to altered tryptophan metabolism, yielding increased concentrations of indolyl 3-acetic acid and indolyl lactate. These results are consistent with the proposal of a purine-driven cell danger response, accompanied by overproduction of epileptogenic and excitotoxic quinolinic acid, large reductions in melatonin synthesis, and gut dysbiosis. In addition, vitamin B6, B12, riboflavin, phenylalanine-tyrosine-tryptophan biosynthesis, pantothenate and pyrimidine metabolism differed significantly in ASD children [10] suggesting that these metabolic abnormalities could underlie several comorbidities frequently associated with ASD [10]. In this work, by using a more sensitive instrument (Orbitrap), allowing us to identify more metabolites, and extending our study to include a greater number of autistic children, we investigated the metabolic pathways that can be influenced by a simultaneous deficit of vitamin B6, vitamin B12 and folic acid (vitamin B9). Moreover, in this investigation, we used a targeted metabolomics platform and different samples in order to provide greater coverage of the metabolites than previous works [10].

Vitamin B6 is the main cofactor of biological reactions such as the synthesis of neurotransmitters and trans-sulfuration, and it is also linked to the metabolism of tryptophan by transforming xanthurenic acid to nicotinic acid. There are three inactive conformations of vitamin B6 (pyridoxal, pyridoxine, pyridoxamine), which are converted into their biologically active form, pyridoxal 5′-phosphate (P5P). Low concentrations of P5P were found in autistic children [11] in combination with a low activity of the pyridoxal kinase enzyme which converts pyridoxal to P5P [12]. Vitamin B12 (cobalamin, Cbl) exists in multiple forms, including methylcobalamin (MeCbl) and adenosylcobalamin (AdoCbl), cofactors for methionine synthase (SM) and methylmalonylCoA mutase, respectively. The metabolically active form of vitamin B12, methylcobalamin (MeCbl) is an essential cofactor for the folate-dependent methylation of homocysteine to methionine by methionine synthase (MS) [13,14]. Autism is associated with a lower vitamin B12 uptake that has been attributed to a dietary intake poor in vitamin B12, dysbiosis in the lining of the intestine that leads to poor absorption of B12, autoimmune antibodies, neurotoxin and heavy metal intoxication that make neurons insensitive to standard doses of B12 [15]. High concentrations of methylcobalamin are required to regenerate neurons and the myelin sheath of the spinal cord, which is necessary for the relief of symptoms in the autism disorder [16]. Folic acid (a form of vitamin B9) is a complex B vitamin that is similar to vitamin B12. Folates are involved in different reactions such as DNA synthesis and repair and in methylation pathways, in particular, it helps prevent the foetus from developing major congenital deformities of the brain or spine, including neural tube defects [17].

Results showed some altered pathways due to three vitamins deficits, including the transmethylation and trans-sulphurization pathways of methionine, justifying the well-known hypometilation of protein and DNA observed in autism [18].

2. Methods

2.1. Participants

Sixty children with idiopathic ASD and 60 typically developing controls (aged 3–8 years) were recruited in Italy and northern Europe. The mean age (±S.D.) of cases and controls was 4.65±0.54 and 5.74±0.45 years, respectively, and the M:F ratio was 42:18 both in ASD and in controls. The diagnosis of autism was formulated according to the Diagnostic and Statistical Manual of Mental Disorders (5th edition) [19]. The research is not a clinical trial and this procedure is in accordance with the Helsinki Declaration of the World Medical Association. Those analyses are considered exploratory.

2.2. Urine collection and metabolite extraction

First-morning urine samples were collected at home by parents using sterile containers untreated with preservatives and were brought to each clinical centre the same morning in wet ice. The urine samples were then frozen, shipped in dry ice, and stored at −80 °C continuously until analysis. Urinary specific gravity was measured by refractometry following centrifugation at 13,000 g for 10 min using a digital refractometer (Euromex Clinical Digital Refractometer RD.5712, NL) previously calibrated with LC–MS grade water. Urine aliquots (200 μl) were mixed with 200 μl of methanol:acetonitrile: water (50:30:20), vortexed for 30 min at maximum speed at 4 °C and then centrifuged at 16,000 g for 15 min at 4 °C. The supernatants were collected for metabolomic analysis. Quality controls (QCs) were obtained from a pooled mixture of 10-μl aliquots of all urine samples and were analyzed every 15 samples.

2.3. Ultra-high-performance liquid chromatograph-mass spectrometry

Twenty microliters of supernatants (three technical replicates) were injected into an ultra-high-performance liquid chromatography (UHPLC) system (Ultimate 3000, Thermo) and run in positive ion mode. A Reprosil C18 column (2.0 mm×150 mm, 2.5 μm – Dr. Maisch, Germany) was used for metabolite separation. Chromatographic separations were achieved at a column temperature of 30 °C and flow rate of 0.2 mL/min. A 0–100% linear gradient of solvent A (ddH2O, 0.1% formic acid) to B (acetonitrile, 0.1% formic acid) was employed over 20 min, returning to 100% A in 2 min and a 6-min post-time solvent A hold. The UHPLC system was coupled online with a mass spectrometer Q Exactive (Thermo) scanning in full MS mode (2 μscans) at 70,000 resolution in the 67 to 1000 m/z range, target of 1×106 ions and a maximum ion injection time (IT) of 35 ms. Source ionization parameters were: spray voltage, 3.8 kV; capillary temperature, 300 °C; sheath gas, 40; auxiliary gas, 25; S-Lens level, 45. Calibration was performed before each analysis against positive ion mode calibration mixes (Piercenet, Thermo Fisher, Rockford, IL, USA) to ensure sub ppm error of the intact mass.

2.4. Metabolomic data processing and statistical analysis

The data were normalized by urinary specific gravity because creatinine excretion may be abnormally reduced in ASD children. Raw files of replicates were exported and converted into mzXML format through MassMatrix (Cleveland, OH, USA), then processed by MAVEN.52 (available at http://genomics-pubs.princeton.edu/mzroll/). Spectrometry chromatograms that were elaborated for peak alignment, matching and comparison of parent and fragment ions, and tentative metabolite identification (within a 2 ppm mass-deviation range between observed and expected results against the imported KEGG database). Results were graphed with Graphpad Prism 5.0 (Graphpad Software). Statistical analyses were performed with the same software. Data are presented as the means±S.D.). *t* Tests, allowing unequal variance, were used to compare changes in mean expression per metabolite between the control and disease groups. A metabolite was considered to be statistically different when $P<.05$.

3. Results

In our previous investigation, the urine samples of children were analyzed by using HILIC columns coupled to a mass analysis using a Q-Tof analyzer, while the statistical analysis was carried out with Metaboanalyst 3.0 software. In this investigation, we increased the number of individuals sampled, collected in different geographical areas, and used a more performant mass spectrometer (Orbitrap). Given that B vitamins are cofactors essential for various biological

reactions related to autism, such as the synthesis of neurotransmitters, the trans-sulfuration process and the metabolism of tryptophan, we focused our attention on the concentrations of B vitamins and analyzed associated processes. Fig. 1 reports the values of reliable indicators of vitamin B deficiencies measured in the urine from autistic children. Pyridoxal 5′-phosphate, the active form of vitamin B6 (Fig. 1A) and an enzyme cofactor involved in the trans-sulfuration process, was altered in the urine from autistic subjects. In the case of vitamin B9, the amount of tetrahydrofolate was reported (Fig. 1B) because it is the final product of the folic acid cycle, which participates in the folate-dependent methylation of homocysteine. Finally, the values of methylmalonic acid (MMA) were determined to assess the state of vitamin B12 (Fig. 1C), which is the most reliable urine test for determining vitamin B12 deficiency. High MMA values are indicative of vitamin B12 deficiency [20].

Regarding the altered metabolomics pathways (Fig. 2**),** the active form of vitamin B6 (P5P) is a fundamental cofactor of cystathionine β-synthase enzyme (CBS) in the bioelimination of toxic homocysteine in the trans-sulfuration process. A low cystathionine concentration in autistic children accounts for the accumulation of homocysteine and serine (upside of Fig. 2) in these patients. As with cystathionine, less cysteine is produced; it also requires vitamin B6 again as a cofactor of the cystathionine γ-lyase enzyme (CSE) for its production. Finally, lower concentrations of taurine as well as glutathione (GSH) were detected in the autistic patients, which may result in less protective action in oxidative processes. Homocysteine, in addition to its irreversible bio-elimination by trans-sulfuration, can be re-methylated to methionine (Fig. 3). Thus, the reduced presence of vitamin B12 in autistic patients blocks this transformation to methionine, which is consistent with the low concentrations recorded in this study. Methionine's methyl group is activated by ATP to form S-adenosyl-L-methionine (SAM), which is a methyl donor for many reactions. A block in the formation of methionine will result in a methyl group deficiency that will prevent SAM formation; this justifies the lower concentrations of SAM in autistic patients. Normally the removal of the methyl group from SAM would lead to the formation of S-adenosyl-L-homocysteine (SAH), which would be immediately converted to homocysteine by the enzyme SAH hydrolase (SAHH), with the removal of the adenosine molecule. This step cannot occur in cases of SAM deficiency. In fact, the concentrations of SAH and adenosine are higher in autistic children. Since the conversion of SAH to homocysteine is reversible, with the equilibrium favoring the formation of SAH, increases in urine homocysteine are matched by an elevation of SAH in most cases. Finally, since vitamin B12 is also involved in the folate cycle (Fig. 4A), the donation of a methyl group from 5-methyltetrahydrofolate (5-methylTHF) to tetrahydrofolate (THF) is strongly reduced, justifying the reduced production of THF recorded in our analysis and the accumulation of 5-methyl-THF. In addition, the concentrations of choline and betaine, the latter of which is transformed into N-N-dimethylglycine (Fig. 4B), functional for the re-methylation of homocysteine, have been altered. Therefore, the concomitant alteration of these metabolic pathways in autistic children significantly reduces the availability of methyl groups and consequently alters the methylation processes.

Finally, Fig. 5 shows a broader picture of the pathways altered in autistic children as related to vitamin B deficiencies. The decreased methionine cycle turnover, related to reduced SAM synthesis, is concomitant with a reduction in cysteine and glutathione synthesis and the two phenomena correlate with three vitamin B deficiencies. In fact, the activation of methionine synthase (MS) requires vitamin B12 as a cofactor, while inhibition of SAH hydrolase activity is induced by oxidative stress resulting from vitamin B6 deficiency. Obviously, the

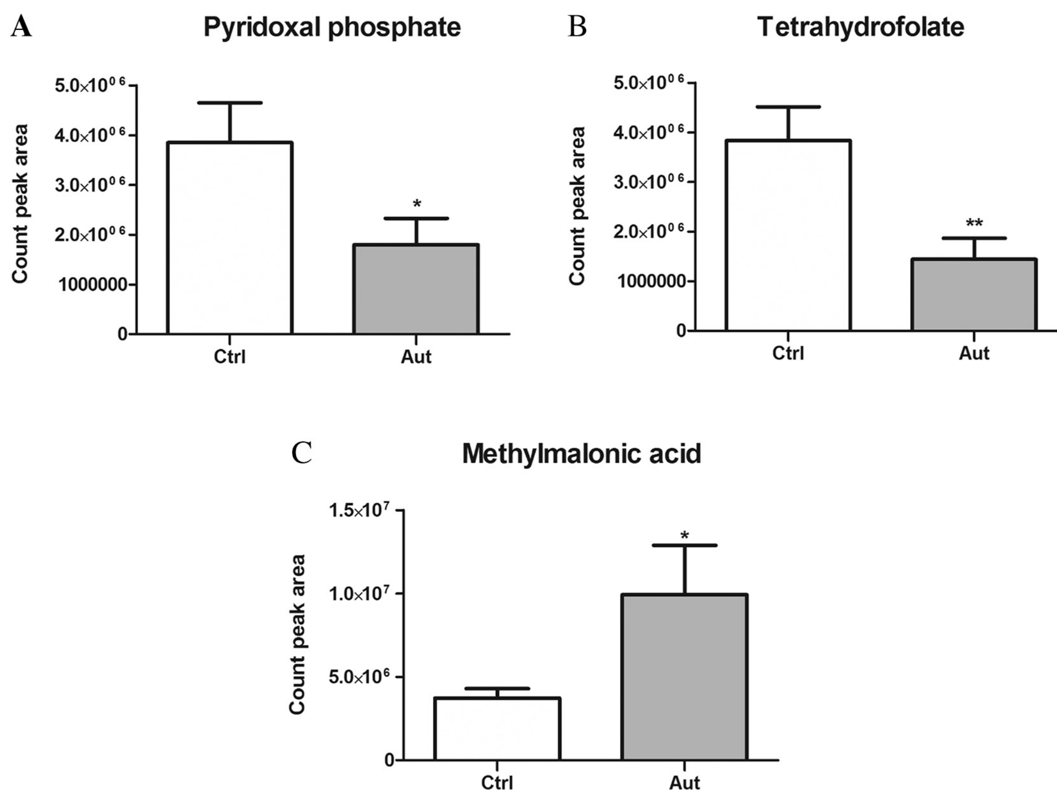

Fig. 1. Reliable indicators of vitamin B deficiency. Panel A reports pyridoxal 5′-phosphate (P5P), the active form of vitamin B6, that is involved in the trans-sulfuration process. Panel B reports tetrahydrofolate (THF), which is the final product of the folic acid cycle that participates in the folate-dependent methylation of homocysteine. Panel C reports methylmalonic acid (MMA), which in the urine test is the most reliable method for determining vitamin B12 deficiency. The results represent the means±S.D. of biological replicates *P<.05, ** P<.01.

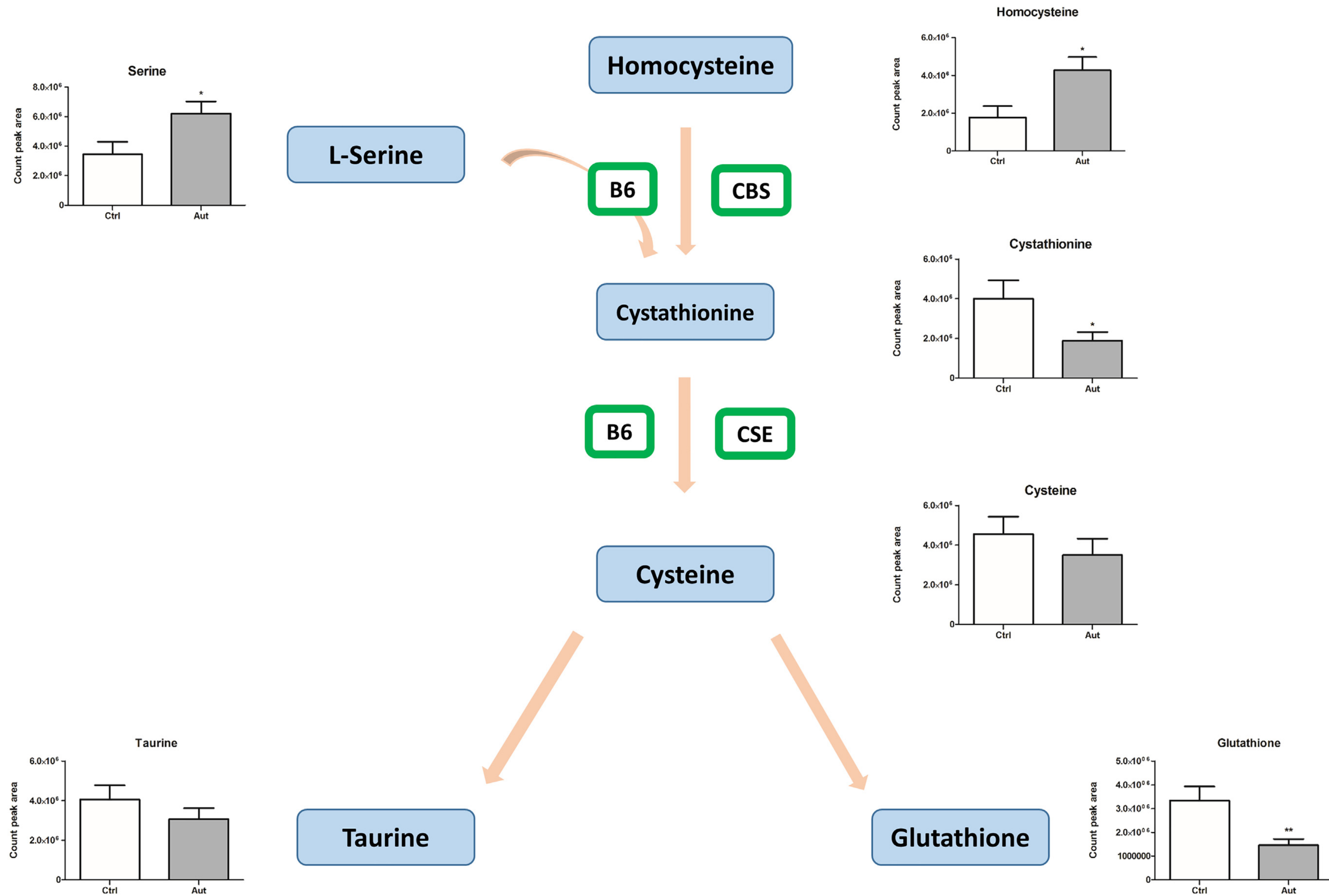

Fig. 2. Quantification of the trans-sulfuration pathway. Vitamin B6 deficiency prevents the correct functioning of the enzyme cystathionine β-synthase (CBS) inducing the accumulation of homocysteine and serine and a reduction in cystathionine concentration in autistic children. Peak areas for each metabolite were normalized by urinary specific gravity. The results represent the means±S.D. of biological replicates *$P<.05$, **$P<.01$.

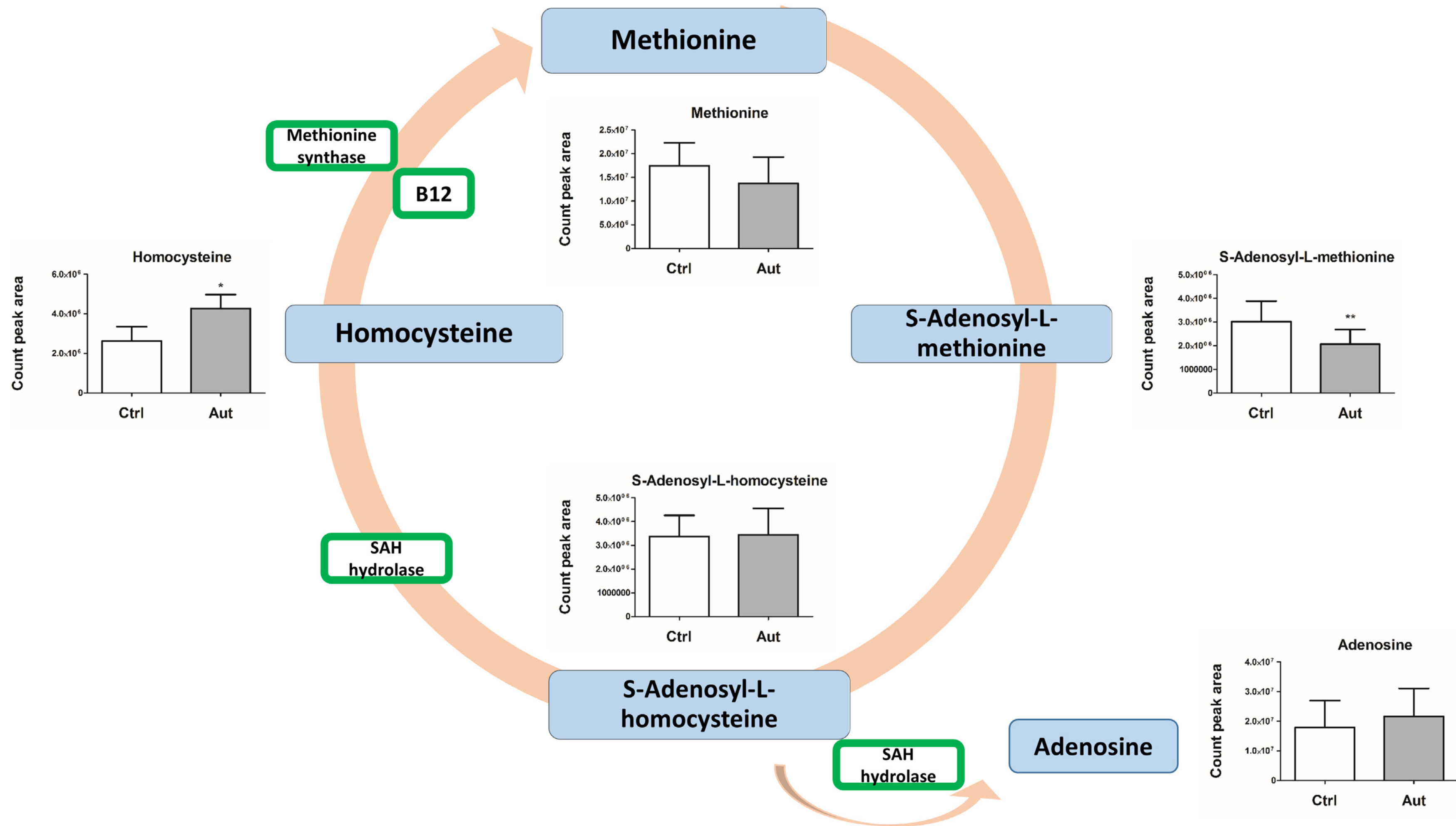

Fig. 3. Quantification of metabolites of methionine cycle. Vitamin B12 deficiency that acts as a cofactor of methionine synthase (MS) induces low S-adenosyl-L-methionine (SAM) concentration caused by a methyl group deficiency. Peak areas for each metabolite were normalized by urinary specific gravity. The results represent the means±S.D. of biological replicates $^{*}P<.05$, $^{**}P<.01$.

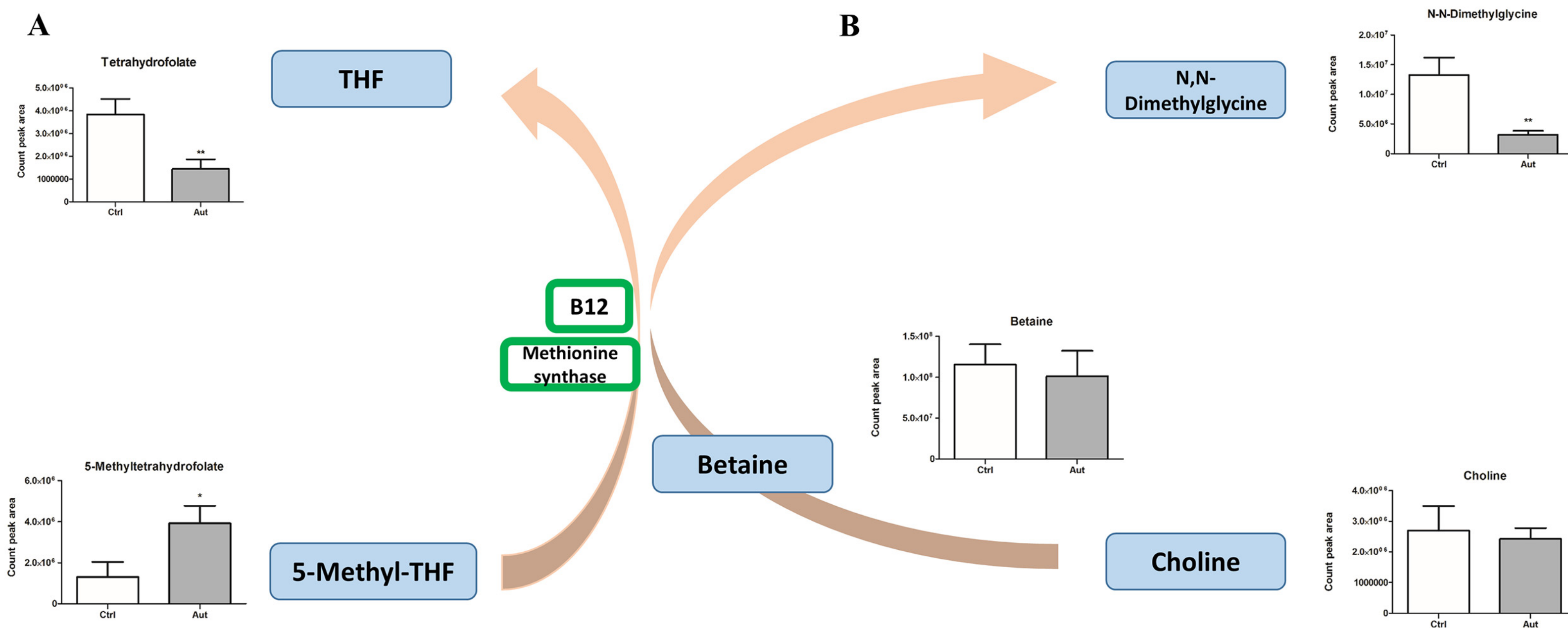

Fig. 4. Panel A shows quantification of the main metabolites included in the folate pathway. Deficiency of vitamin B12 induces a blockage in the folate cycle carrying an accumulation of 5-methyltetrahydrofolate (5-methylTHF). Panel B shows the metabolites involved in the transformation of betaine to N-N dimethylglycine. Peak areas for each metabolite were normalized by urinary specific gravity. The results represent the means±S.D. of biological replicates $^*P<.05$, $^{**}P<.01$.

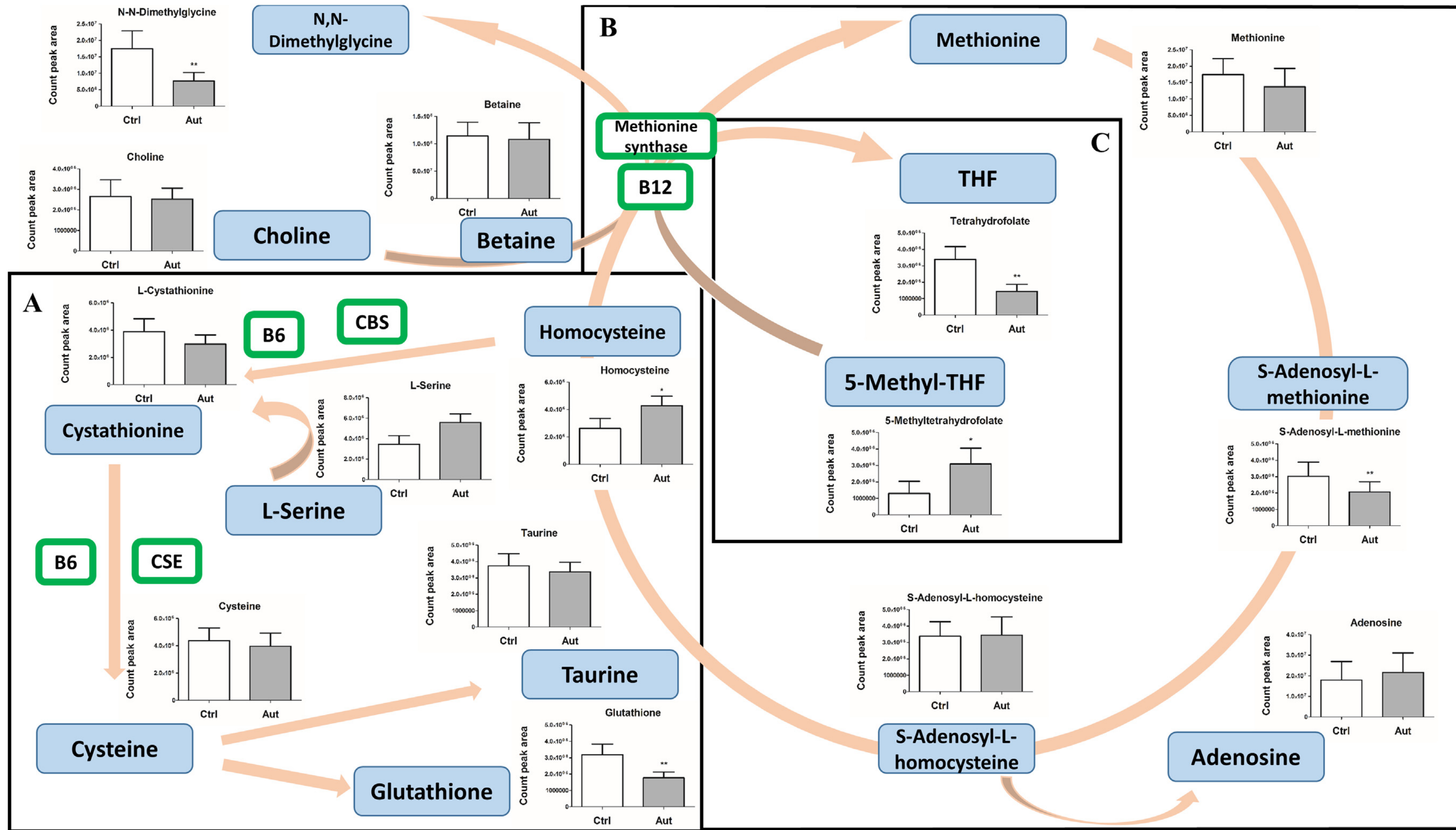

Fig. 5. Overview of the previously discussed cycles. Panel A represents the trans-sulfuration process in which homocysteine is eliminated and transformed into antioxidant precursors such as glutathione (GSH). Panel B shows the methionine cycle essential for the methylation process with the formation of S-adenosyl-L-methionine (SAM) as the main methyl donor. Panel C shows folate cycle: 5-methyltetrahydrofolate (5-methylTHF), which cannot provide the methyl group that is essential for the methylation reaction. The values were obtained from the urine of 60 autistic children compared to 60 matched controls (age range 3–8 years). The results represent the means±S.D. of biological replicates $^{*}P<.05$, $^{**}P<.01$.

consistent decrease in the SAM / SAH ratio is related to a lack of vitamin B6.

4. Discussion

The present study reports significant urinary metabolic differences between healthy children and those with idiopathic ASD, related to reduced concentrations of vitamin B6, folic acid (vitamin B9) and vitamin B12, which can explain the alteration of protein and DNA methylation of autistic children, reported in literature [18,21]. It is interesting to note that dietary vitamin B deficiencies complicate the care of critically autistic children [22]. Using a more accurate and sensitive technical approach, it has been possible to achieve a broader picture of the pathways altered in autistic children as related to vitamin B deficiencies (Fig. 5). Pyridoxal 5′-phosphate (P5P) is the active form of vitamin B6 that is used as a cofactor, together with the enzyme cystathionine β-synthase, for the condensation of serine and homocysteine to form cystathionine. This is further converted to cysteine by completing the process called trans-sulfuration, which again requires vitamin B6 as a cofactor of the enzyme cystathionine γ-lyase (CSE) [23]. Thus, lower concentrations of vitamin B6 block cysteine formation and result in the accumulation of the toxic homocysteine (Fig. 5A). The consistent decrease in plasma methionine and cysteine concentrations observed in autistic children suggested that cysteine may be an essential amino acid for these children [24]. More importantly, cysteine is the source of both taurine and hydrogen sulfide (H2S), in addition to being the essential amino acid constituent in the functional (CXXC) motif of the major cellular antioxidant families, which includes reduced glutathione (GSH). Since the intracellular GSH-GSSG redox status provides the essential intracellular reducing environment required for normal immune function, detoxification capacity, redox-sensitive enzyme activity, and membrane redox signaling [25–27] it is not surprising that autistic children exhibit systemic evidence of oxidative stress. Oxidative stress occurs when antioxidant defense mechanisms fail to counterbalance and control reactive oxygen species generated from endogenous oxidative metabolism or from pro-oxidant environmental exposures, such as vitamin B6 deficiency. Several recent reviews and research studies lend support to the hypothesis that redox imbalance and oxidative stress may be a contributing factor to autism pathology [28–30]. This theory has sparked a new field of research focused on optimizing glutathione concentrations as a way to treat autism [23]. In agreement, a decrease in glutathione concentrations was also recorded in children with autism but consequent to increased environmental mercury concentrations, bad gut bacteria, and poor diet [29], supporting that GSH reduction may result from vitamin B6 deficiency. Paşca presented a possible connection between autism spectrum disorders (ASD) and high concentrations of homocysteine for the first time in 2006 [31]. Recent studies have confirmed that high concentrations of homocysteine in biological fluids are related to oxidative stress and are generally associated with neuropsychiatric disorders such as autism [29,32]. Homocysteine leads to increased excitatory glutamatergic neurotransmission in different areas of the brain where neuronal damage results from excess Ca2+ influx and reactive oxygen generation [33], which results in increased oxidative stress. Thus, vitamin B6, B12 and B9 deficits act in a syntonic manner. Furthermore, various studies have suggested that homocysteine might regulate the function of other neuromodulators, such as acetylcholine [34] dopamine and serotonin [35], in agreement with our previous investigations of altered tryptophan metabolism in autism [10]. These results further support the idea that more than one metabolomics pathway is altered in autism, which may not be correlated to concomitant specific gene mutations. The accumulation of homocysteine is also a consequence of vitamin B12 deficiency (Fig. 5B) [36] and its high levels were associated with oxidative stress and DNA hypomethylation [37]. The re-methylation of homocysteine to methionine requires a methyl group provided by 5-methyltetrahydrofolate, through methionine synthase (MS) and the cofactor vitamin B12 [38]. The reduced activity of MS leads to lower concentrations of tetrahydrofolate (folate cycle), as reported in Fig. 5C. A deficit of methionine synthase or a deficiency of B12 may lead to a so-called "methyl-trap" of tetrahydrofolate (THF), in which 5-methylTHF is not converted into THF, which does not provide methyl groups for methylation reactions [39]. The strict relationship between folic acid and vitamin B12 is shown in Fig. 5C. For this reason, folate and vitamin B12 deficiencies have been implicated in the manifestation of various clinical disorders [40] and have been shown to arrest children's development [41]. Children with autism or a pervasive developmental disorder very often exhibit feeding difficulties [42]. Although folate is required for metabolic processes and neural development, there is a debate about its protective effect against ASD. Some authors have concluded that a folic acid deficit is associated with an increased risk for autism, however higher concentrations can give negative neurocognitive development outcomes [43]. Transformation of homocysteine to methionine requires vitamin B12 as a cofactor of methionine synthase (MS). Since the activity of MS determines the relationship between S-adenosyl-L-methionine (SAM) and the methylation inhibitor S-adenosyl-L-homocysteine (SAH), vitamin B12 deficiency can influence hundreds of SAM-dependent methylation reactions [16]. There was a significant decrease in urinary methionine and S-adenosyl-L-methionine (SAM) concentrations, which is the major methyl donor, while adenosine and S-adenosyl-L-homocysteine (SAH) concentrations increased. Low concentrations of methionine and SAM in combination with increased concentrations of SAH and adenosine have been previously demonstrated to be associated with reduced cellular methylation [44]. In particular, a decrease in the SAM / SAH ratio has been associated with hypomethylation of DNA, RNA, proteins, phospholipids and neurotransmitters [45] exerting an important epigenetic effect. Thus, our investigation and reconstruction of altered metabolic pathways we shed light it may be one possible explanation of the relationship between ASD and DNA hypomethylation [8,9]. In this regard, we are currently investigating the correlation between the decreased SAH/SAM ratios recorded in subgroup of autistic children with the degree of DNA methylation recorded in their lymphocytes (data not shown). This will better validate what previously reported by other authors [8,9] that the decreased methylation process, related to vitamin B deficiencies, results in hypomethylation of DNA in autism.

What is common in most autistic patients is a presence of intestinal dysbiosis associated with chronic inflammation, an increased prevalence of gastrointestinal inflammation and increased mucosal permeability in the upper and lower intestines [46] that may be mediated by innate microglial activation and proinflammatory cytokines [47]. The inflammatory response is increased when GSH concentrations are low [48], as here recorded. Moreover, chronic inflammation depletes GSH, which further promotes a self-perpetuating cycle that could exacerbate gastrointestinal, central nervous system inflammation, associated with autism, and exacerbate oxidative stress [46].

As a final consideration, by using mass spectrometer technique we found a concomitant deficiency of all three B vitamins in most autistic children, suggesting that a common intestinal cause could exists.

Authors' contributions

LZ was responsible for study design, study coordination, and manuscript writing. FG was responsible for data collection and statistical analysis of data. AB was responsible for HPLC-MS analysis and quantification of urine metabolites and data collection and interpretation. All authors read and approved the final manuscript.

Acknowledgments

LZ, AB and FG are supported by mobility studentship funds and post-doctoral research grant by the Interuniversity Consortium for Biotechnologies (CIB). We gratefully acknowledge the patients, controls, and family members who participated in this study.

Conflict of interest statement

None of the authors has any financial conflict of interest.

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