IL-6, homocysteine, and autism spectrum phenotypes: an investigation among adults with autism spectrum disorder and their first-degree relatives

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Abstract

Background. The importance of recognizing different kinds of autism spectrum presentations among adults, including subthreshold forms and the broad autism phenotype (BAP), has been increasingly highlighted in recent studies. Meanwhile, the possible involvement of immune system deregulation and altered methylation/trans-sulfuration processes in autism spectrum disorder (ASD) is gaining growing attention, but studies in this field are mainly focused on children. In this framework, the aim of this study was to compare plasmatic concentrations of IL-6 and homocysteine (HCY) among adults with ASD, their first-degree relatives, and healthy controls (CTLs), investigating also possible correlations with specific autism symptoms.
Methods. Plasma concentrations of IL-6 and HCY were measured in a group of adult subjects with ASD, their first-degree relatives (BAP group), and healthy controls (CTL). All participants were also evaluated with psychometric instruments.
Results. IL-6 and HCY concentrations were significantly higher in the ASD group than in CTLs, while BAP subjects reported intermediate results. Significant correlations were reported between biochemical parameters and psychometric scales, particularly for the dimension of ruminative thinking.
Conclusions. These findings support the hypothesis of a key involvement of HCY-related metabolism and immune system alteration in autism spectrum pathophysiology. HCY and IL-6 seem to show different associations with specific autism dimensions.

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Introduction

Autism spectrum disorder and the broad autism phenotype

Autism spectrum disorder (ASD) is a neurodevelopmental condition characterized by an early onset of symptoms, usually in childhood. The main symptoms feature impairment in social communication and interactions, narrow interests, and repetitive behaviors.[1] Among ASD individuals, the severity of symptoms may vary greatly, including the presence or not of intellectual impairment and language development alterations.[2,3] Although most of the studies on ASD have been focused on children, recent research is also examining clinical ASD presentations in adulthood, but scant literature evaluated possible biochemical correlates specific to ASD in adult life.[4] In particular, several authors highlighted how milder forms of autism may remain underdiagnosed in childhood, and patients may come to clinical attention only during adult life, after the development of other psychiatric disorders in comorbidity.[2,3] In addition, increasing interest has been paid to subthreshold forms of ASD, which have been first investigated among first-degree relatives of ASD probands.[5,6] In particular, this population was reported to show personality traits and neurostructural correlates similar to those of their affected relatives, although less severe.[7,8] Starting from these considerations, the presence of a broad autism phenotype (BAP) was stressed in the literature.[2-4,7,9] Although BAP prevalence is higher among close relatives of ASD people than among general population,[10,11] the presence of subthreshold autistic traits seems to be continuously distributed from the general to the clinical population, being particularly higher in high-risk groups.[3] Moreover, BAP presence seems to be associated with an increased risk of psychiatric disorders, suicidal thoughts, and behaviors, further increasing the importance of detecting these conditions also when clinically subthreshold.[3,9] While the presence of neurostructural and neurofunctional alterations in BAP has recently been highlighted by neuroimaging evidences,[8,9,12] biochemical research on this specific matter is still in its infancy.[4,12]

Cytokines studies in ASD patients

Since the 1970s, the frequent presence of somatic conditions such as diabetes, inflammatory bowel diseases, allergies, and asthma was reported among ASD children, leading many authors to focus on immune system alterations in ASD.[4,13-17] In the last decades, growing interest has been paid on cytokines and interleukins deregulation in ASD. Cytokines are considered particularly promising as potential biomarkers in this field because they are a source of information about the state of the immune system relatively simple to measure, and, on the other hand, they are also able to directly affect the central nervous system (CNS).[4,14] In this framework, Masi et al have stressed how cytokines may affect CNS through the induction of behavioral changes as a part of the response against infection and immune challenges. Among the others, IL-6 is one of the most investigated cytokines in psychiatry and particularly in mood disorders.[18] In ASD children, the presence of increased peripheral levels of pro-inflammatory cytokines, including IL-6, was reported by several studies.[19-21] Several meta-analyses are available on this topic, generally reporting increased levels of pro-inflammatory cytokines and decreased levels of anti-inflammatory ones in ASD children, although stressing the heterogeneity of the available studies.[19-21] IL-6 was one of the cytokines more constantly reported to be increased among ASD children, and some findings suggested also its possible correlation with the severity of ASD symptoms.[4,22,23] Despite that, few studies focused on altered IL-6 levels in adult samples of ASD subjects. The investigation in this field seems to be limited to two studies, which reported a positive, but nonsignificant trend toward higher levels of IL-6 in postpuberal or adult ASD patients than in controls.[24,25] Similarly, poor literature investigated IL-6 levels among BAP subjects, while first-degree relatives of ASD children have instead been considered as a control group in some studies.[4] Other authors found significantly higher levels of IL-6 in ASD children than in unrelated siblings of other ASD individuals,[26] while Napolioni et al[27] did not find instead significant differences between ASD patients and their typically developed siblings.

Homocysteine alteration in ASD

Recently, a growing number of studies pointed out the interest of homocysteine (HCY) related metabolism in the pathophysiology of neurodevelopmental disorders. HCY is known to be involved in many somatic and neuropsychiatric conditions, ranging from stroke and thrombosis[28] to mood disorders, schizophrenia, ASD, and Alzheimer's disease.[28-31] HCY, through the trans-methylation pathway of its metabolism, is, together with S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH), an indicator of methyltransferase activity and of the methylation state of different substrates, including DNA.[32,33] In addition, through the trans-sulfuration pathway, HCY metabolism may influence glutathione synthesis and redox homeostasis: elevated HCY levels are linked to higher oxidative states.[34-36] Noticeably, both increased DNA methylation and altered redox balance have been reported among ASD subjects.[36,37] The ratio between the methyl donor SAM, a source of HCY, and the methylation inhibitor SAH (or SAM/SAH), is considered to be an indicator of cellular methylation capacity and was reported to be altered in ASD.[37] HCY accumulation may be linked to genetic factors, impaired metabolic mechanisms, or also to an insufficient intake, altered absorption, or metabolic use of vitamin B6, B12, and folate.[28,36]

Research on altered HCY concentration in ASD mainly targeted children and, in a few cases, adolescents. In particular, James et al[38] reported in ASD children reduced levels of HCY, methionine, SAM, cystathionine, cysteine, and glutathione as well as higher levels of SAH, adenosine, and oxidized glutathione when compared with controls. On the basis of this data, the authors hypothesized the presence of a diminished SAH hydrolysis, linked to increased adenosine, consequently leading to a decreased HCY synthesis.[38] On the other hand, further studies reported the presence of increased HCY levels in plasma and serum of ASD children.[28,39-43] In particular, a recent meta-analysis of 31 studies[43] showed that HCY levels seem to be increased in both serum and plasma of children with ASD. However, other studies failed to find this association.[44-46] Several studies on urine samples also reported higher levels of HCY in ASD patients.[28,42,47-50] Another work showed that not only urinary levels of HCY were higher in ASD individuals compared to controls but seemed also to be correlated with impaired communication skills. However, no correlation was found with socialization deficits and repetitive/restricted behaviors as measured by revised Autism Diagnostic Interview (ADI-R).[50]

Han et al[36] compared levels of a wide set of metabolites of trans-sulfuration metabolism, such as HCY, cysteine, total glutathione, reduced (GSH), and oxidized glutathione (GSSG) between ASD children and controls. These authors found higher levels of HCY and GSSG, as well as lower levels of cysteine, total glutathione, GSH, and GSH/GSSG ratio in ASD subjects than in controls. Furthermore, HCY levels were positively correlated with the scores reported by the patients at the Childhood autism rating scale (CARS).[36] Other studies included also unaffected relatives in their investigation. Melnyk et al[37] compared a group of ASD children, their unaffected siblings and controls, failing to find a significant difference for circulating levels of HCY, folate, and vitamin B12, but reporting a higher oxidative state in ASD children and lower levels of methionine, SAM and SAM/SAH ratio, together with a lower percentage of DNA 5-methylcytosine and increased levels of SAH form of cysteine (Cys-SS). Intermediate levels of SAH and free glutathione were reported in the sibling group. Main et al[46] did not find any significant difference in cytokinesis-block micronucleus cytome (CBMN-cyt), HCY, and B vitamins among ASD children, their unaffected siblings and controls, hypothesizing that genomic instability may not be considered as a feature of ASD.[46] Other authors examined the levels of HCY, methionine, cysteine, SAM, SAH, glutathione, 5-methylcytosine, and total cytosine in DNA among mothers and fathers of ASD children, comparing them with control mothers. Parents of ASD subjects reported increased levels of HCY, SAH, and GSSG, but lower GSH levels and GSH/GSSG or SAM/SAH ratio.[51,52] Globally, despite the available literature that seems to confirm the presence of impaired methylation/trans-sulfuration pathways and increased HCY levels in autism spectrum conditions, studies are still limited to samples of children.

Aims of this work

As reported above, despite an increasing number of psychopathological studies have stressed the importance of investigating autism spectrum features among adults, especially for improving our understanding of those milder forms that may remain underdiagnosed during childhood, most of the available biochemical research in autism field is focused on children.[4,12] Biochemical correlates of the same condition may greatly vary from childhood to adulthood, as well as their consequent potential as biochemical markers. In this framework, among other biochemical parameters, investigating

IL-6 and HCY in older subjects could be of particular interest, considering that epigenetic and redox status alteration, as well as immune system activity, could vary during lifetime in response to environmental stressors.[36,37]

In light of the above-mentioned literature, the present study aimed to compare circulating levels of IL-6 and HCY among adult ASD subjects (ASD group), their adult first-degree relatives (BAP group), and unrelated healthy controls (CTL group), in order to evaluate potential biochemical correlates of ASD in adult life as well as similarities and differences between subthreshold and full-threshold autism phenotypes. Specific correlations between biochemical parameters and autism spectrum symptoms and traits, as measured by psychometric scales, were also evaluated. We hypothesized to find increased levels of IL-6 and HCY in adults with ASD, while intermediate levels between patients and CTLs are expected in the relatives' group. We also hypothesized to find significant correlations between biochemical parameters' concentrations and the scores reported on psychometric scales.

Methods

Recruitment procedures

Participants were recruited among subjects followed at the Psychiatric Clinic of University of Pisa. A group of adult patients with ASD (ASD group) was recruited among in- and out-patients. For each subject, the enrollment of one nonaffected relative (parent or adult sibling) was also requested in order to recruit the relatives' group (BAP group). In order to be included in the study, patients must be aged between 18 and 65 years and have received a clinical diagnosis of ASD. The ASD diagnosis was clinically confirmed (if yet received in childhood or in other settings) or performed according to DSM-5 criteria by trained mental health professionals at the time of the recruitment. Exclusion criteria were: the presence of major intellectual impairment, with a consequent inability to fill out the psychometric instruments; a diagnosis of schizophrenia or of substance use disorder; the presence of other relevant neurological/medical disease. In addition, subjects in the BAP group were not included also if they reported a diagnosis of ASD. A group of adult controls without a diagnosis of psychiatric disorders was recruited on a voluntary basis. All subjects received clear information about the study and had the opportunity to ask questions before providing a written informed consent. This work was conducted in accordance with the declaration of Helsinki, and all procedures were approved by the local ethical committee.

Psychometric instruments

The Structured Clinical Interview for DSM-5 disorders (SCID-5) was used for evaluating the presence of mental disorders.[53] In addition, the Adult Autism Subthreshold Spectrum (AdAS Spectrum) and the Ritvo Autism and Asperger Diagnostic Scale (RAADS-14) were used for measuring the wide range of autism spectrum symptoms. In addition, the Ruminative Response Scale (RRS) was used for measuring the specific dimension of ruminative thinking. Ruminative thinking is a feature closely associated with the autism spectrum: although it was reported to be trans-diagnostic, some authors hypothesized that the tendency toward rumination may be underlain by full-threshold or subthreshold autistic traits also in different psychiatric conditions. Considering that the recent literature highlighted a role of ruminative thinking

in worsening psychopathological picture, we chose to investigate also this specific dimension in our sample.[2,54] Finally, in order to assess how the reported symptoms of the autism spectrum may impact on subjects' adjustment, we included in the evaluation the Work and Social Adjustment Scale (WSAS), which is tailored for measuring the levels of adjustment with respect to the reported symptoms.[55] In particular, for the aims of this work, subjects were specifically asked to fill out the WSAS referring only to the symptoms investigated by the other scales and not to eventual other symptoms.

The Adult Autism Subthreshold Spectrum

The AdAS Spectrum is an instrument developed by Dell'Osso et al[3] aiming to evaluate the broad range of subthreshold and clinical manifestations of the autism spectrum during lifetime among adults without intellectual impairment or language development alteration. The instrument is composed of seven domains: *Childhood/adolescence, Verbal communication, Nonverbal communication, Empathy, Inflexibility and adherence to routine, Restricted interests and rumination,* and *Hyper-hypo reactivity to sensory input.* All items feature a dichotomous answer (yes/no). The instrument showed an excellent reliability (Kuder–Richardson's coefficient = 0.964) according to the validation study.[3]

The Ritvo Autism and Asperger Diagnostic Scale

The RAADS-14 is a shortened version of the RAADS. The instrument was developed for assessing concisely the main symptoms of autism and features 14 items with answers organized on a Likert scale. The domains of the scale are *Mentalizing deficits, Social anxiety,* and *Sensory reactivity.* The RAADS-14 showed excellent internal consistency in the validation study, with a Cronbach's alpha = 0.90.[56]

The Ruminative Response Scale

The RRS is a questionnaire tailored to assess the specific dimension of ruminative thinking. Answers are organized on a Likert scale, and divided into three dimensions: *Brooding, Reflection,* and *Depression.* In the validation study, the RRS showed an excellent internal consistency, with Cronbach's alpha = 0.89.[54]

The Work and Social Adjustment Scale

The WSAS is an instrument composed of five items organized in a 9-point Likert scale, aiming to evaluate how much symptoms affect social and work functioning (*Work, Home management, Social leisure activities, Private leisure activities, Ability to form and maintain close relationships*). Scores range from 0 to 40: higher scores indicate a greater impairment. The questionnaire is widely used in the literature and showed good internal consistency in the validation study (Cronbach's alpha ranging from 0.80 to 0.90).[55]

Biochemical evaluations

A peripheral blood sample was collected in the morning from each participant. Subjects were requested to fast for 12 hours before the blood draw. In order to separate platelet-rich plasma (PRP) from other cellular elements, the blood samples, collected in K_3EDTA vacutainer tubes, were centrifuged for 15 minutes at 150 g. Subsequently, the PRP aliquots were centrifuged again in Falcon tubes for 15 minutes at 1500 g, thus obtaining platelet-poor plasma (PPP) samples. All operations were performed at room temperature. PPP samples were transferred in high-quality, low-binding protein

Eppendorf Safe-Lock test tubes, and maintained at −80°C until the day of the assay. Concentrations of IL-6 and HCY in the PPP were determined by means of enzyme-linked immunosorbent assays (ELISA). In particular, IL-6 levels were assessed with a sandwich ELISA kit (Picokine IL-6 assay, Boster Biological Technology, Pleasanton, CA). First, the defrosted aliquots of PPP were diluted in a sample diluent buffer as requested by the instructions. The kit featured a first anti-IL-6 monoclonal antibody, a second biotinylated antibody, and a streptavidin-biotin-peroxidase complex. At the end of the reactions, the plate absorbance was read at $\lambda = 450$ nm by a plate reader spectrophotometer. The standard calibration range for calculating the calibration curve was 4.69–300 pg/mL. The calibration curves were calculated through a 4-parameter logistic regression, and IL-6 concentrations in unknowns were interpolated as pg/mL. This method featured a determination limit of 0.3 pg/mL, thus being associated with a high sensitivity. The assessment of HCY levels was instead performed by means of an indirect competitive ELISA kit produced by ImmuSmol (Bordeaux, France). Following the kit instruction, PPP samples were incubated with an enzyme reaction mixture, featuring the S-adenosyl-L-homocysteine hydrolase enzyme and its substrate adenosine/dithiothreitol (DTT), before performing the ELISA procedure: this step was requested for transforming the whole amount of HCY into SAH, thus avoiding matrix effects that may happen when HCY is measured directly. The ELISA microplate of the kit was precoated with SAH. The competitive assay was performed through an incubation step with a monoclonal anti-SAH mouse antibody, followed by another step with a second biotinylated anti-mouse antibody. The revelation step featured the addition of a biotin-streptavidin complex coupled to horseradish peroxidase (HRP), and then of the HRP substrate, 3,3′,5,5′-tetramethylbenzidine (TMB). As in the case of IL-6, the plate absorbance was read at $\lambda = 450$ nm by a plate reader spectrophotometer. The calibration curve, calculated by a 4-parameter logistic regression, ranged from 2 to 50 μM.

Statistical analysis

The use of nonparametric tests was preferred because the normality tests and variance homoscedasticity were not respected in our sample. For comparing biochemical parameter concentrations, a Kruskal–Wallis one-way analysis of variance was performed. Mann–Whitney tests were used for the comparison of IL-6 and HCY concentrations depending on the presence of comorbid conditions or pharmacological treatments. A Spearman's correlation coefficient was calculated for evaluating the correlations between biochemical variables and the scores reported on psychometric instruments. All the analyses were performed using SPSS version 24 (IBM Corp., Armonk, NY, 2016) and GraphPad Prism (Version 7.0, San Diego, CA). The calibration curves and the regression analysis for the biochemical assays were calculated through GraphPad.

Results

Comparison of biochemical parameters among groups

Sociodemographic characteristics of the samples, as well as clinical features such as comorbidities and use of pharmacological treatments, were reported elsewhere.[57] When comparing IL-6 and HCY levels among groups, both the parameters were found significantly higher in the ASD group than in the CTL group, while BAP group showed intermediate concentrations, not significantly different from the other groups (see Table 1). In addition, 29.17% (n = 7) of ASD subjects, 8.33% (n = 2) of BAP ones, and 0% (n = 0) of the CTLs showed HCY values above 15 μM, which is considered the threshold level of Hyper-HCY (while optimal values are considered lower than 10 μM).[58] No significant differences were reported for the comparison of IL-6 and HCY concentrations depending on the presence of comorbid conditions or pharmacological treatments.

Correlations between biochemical parameters and scores reported on psychometric scales

When considering the correlations between biochemical parameters and psychometric scales, we found that the AdAS Spectrum total score and all AdAS Spectrum domains, with the exception of *Empathy*, were significantly and positively correlated with HCY concentrations, but not with IL-6 (see Table 2). RAADS-14 total and

Table 1. Comparison of HCY and IL-6 Levels Among Groups

	ASD	BAP	CTL		
	(n = 24)	(n = 24)	(n = 24)		
	(Mean ± SD, Mean rank)	(Mean ± SD, Mean rank)	(Mean ± SD, Mean rank)	H	P
IL-6 (pg/mL)	21.012 ± 9.787, 24.54	17.993 ± 6.008, 22.31	12.964 ± 5.054, 13.15	7.30	**.026***
HCY (μM)	12.939 ± 8.485, 40.18	10.001 ± 3.520, 36.95	7.507 ± 2.339, 23.96	9.03	**.011***

Abbreviations: ASD, autism spectrum disorder; BAP, broad autism phenotype; CTL, controls; HCY, homocysteine; IL-6, interleukin 6.
*ASD > CTL, P < .05.
The statistically significant values are reported in bold.

Table 2. Correlations Between AdAS Spectrum Scores and Biochemical Parameters in the Whole Sample

	Childhood/ adolescence	Verbal commun.	Nonverbal commun.	Empathy	Inflexibility and adherence to routine	Restricted interests and rumination	Hyper-hypo reactivity to sensory input	AdAS total score
IL-6 (pg/mL)	.229	.202	.294	.175	.322	.284	.235	.269
HCY (μM)	**.332****	**.260***	**.274***	.202	**.345****	**.385****	**.280***	**.345****

Abbreviations: HCY, homocysteine; IL-6, interleukin 6.
*P < .05;
**P < .01.
The statistically significant values are reported in bold.

Table 3. Correlations Between RAADS-14 Scores and Biochemical Parameters in the Whole Sample

	Mentalizing deficit	Social anxiety	Sensory reactivity	RAADS-14 total score
IL-6 (pg/mL)	**.346***	.124	.179	.267
HCY (μM)	**.392****	**.280***	.216	**.358****

Abbreviations: HCY, homocysteine; IL-6, interleukin 6.
*P < .05;
**P < .01.
The statistically significant values are reported in bold.

Table 4. Correlations Between RRS Scores and Biochemical Parameters in the Whole Sample

	Reflection	Brooding	Depression	RRS total score
IL-6 (pg/mL)	**.409***	**.475****	**.509****	**.483****
HCY (μM)	.257	**.469****	**.422***	**.403****

Abbreviations: HCY, homocysteine; IL-6, interleukin 6.
*P < .05;
**P < .01.
The statistically significant values are reported in bold.

domain scores, with the exception of *Sensory reactivity*, were also significantly and positively correlated with HCY concentrations. A significant positive correlation was found between the *Mentalizing deficit* domain and IL-6 levels (see Table 3). Both IL-6 and HCY were reported to be significantly and positively correlated with RRS total and all domain scores, with the exception of the *Reflection* domain, which was significantly correlated only with IL-6 (see Table 4). Finally, WSAS total score and most of the single-item scores were positively correlated with both HCY (with the exception of *Social leisure activities* and *Close relationships*) and IL-6 (with the exception of *Home management*) (see Table 5).

Discussion

Differences in IL-6 and HCY levels among groups

The aim of this work was to compare circulating levels of HCY and IL-6 among subjects with ASD, their first-degree relatives (BAP group), and CTLs. First, the concentration ranges reported here for the investigated biochemical variables were globally in line with those reported in previous human studies, although in the framework of a high variability between different researches.[39,42,55,59] According to our data, both HCY and IL-6 levels were significantly higher in ASD subjects than in CTLs, while the BAP group reported intermediate levels. Several previous studies highlighted increased pro-inflammatory cytokines in ASD children[4]: in this population, IL-6 is one of the cytokines most constantly reported to be

elevated.[4,19,59] While our results seem in line with this data, the few available researches in ASD adults reported only a trend toward increased IL-6 levels, failing to find significant differences between ASD and CTLs.[25,60] Noticeably, our findings are somewhat in line with previous investigations that stressed, in adult subjects with ASD, increased levels of ciliary neurotrophic factor (CNTF), with respect to typically developed subjects or No ASD individuals with intellectual disability. CTNF is a neurotrophin that may act as signal of neuronal damage, and its increased levels in ASD may support the link between autism spectrum and neuroinflammation.[61] When considering BAP, the intermediate levels reported here could be considered somewhat in line with previous literature. In particular, IL-6 levels were reported to be higher in pregnant mothers of ASD patients than in controls mothers,[4,62] while one study from Manzardo et al even reported lower IL-6 levels in ASD children than in siblings of other ASD patients.[26] IL-6 is known to be involved in promoting sickness behavior and its increased expression has been associated with neurodegeneration.[63,64] Increased IL-6 levels have been observed not only in autoimmune disorders, but also in some neurodegenerative conditions such as Alzheimer's disease, and in different kinds of mental disorders.[63] Several authors hypothesized a key role of IL-6 in mediating the communication between immune system and CNS. Our data, highlighting increased levels of IL-6 in adult ASD patients, are in line with the reported alteration of immune and inflammatory activity among subjects with ASD, and may support the hypothesis of intertwined relationships between immune system and CNS, which may affect each other activities.[4]

Considering HCY, our findings seem to confirm in adult sample results from previous studies, which reported increased HCY concentrations in children or adolescents with ASD,[42,43] although not all the authors replicated this result.[46,65] It is worth noting that our data also revealed a mean HCY level above the optimal one (<10 μM) among ASD subjects and a borderline mean value among BAP ones (12.939 ± 8.485 and 10.001 ± 3.520, respectively). A 27% rate of subjects with hyperhomocysteinemia (HCY levels > 15 μM) was also revealed in the ASD group, together with a 8.33% rate in the BAP group, with respect to a 0% rate in CTLs.[58] Among children, who usually show lower levels of HCY than adults, subjects with ASD reported values above the threshold of 15 μM only in some of the available studies.[28,39,58] The report of intermediate HCY levels in BAP subjects is in line with the continuum between ASD and BAP features stressed in psychopathological studies[9] and in the few available biochemical investigations. In particular, a study from James et al[51,52] highlighted, among parents of ASD children, increased HCY, SAH, and GSSG levels, together with lower GSH levels, GSH/GSSG and SAM/SAM ratio, and DNA hypo-methylation, with respect to parents of non-ASD children. Intermediate levels of SAH in siblings of ASD children with respect to the probands and the controls were also reported by Melnyk

Table 5. Correlations Between WSAS Scores and Biochemical Parameters in the Whole Sample

	Work	Home management	Social leisure activities	Private leisure activities	Close relationships	WSAS total score
IL-6 (pg/mL)	**.432***	.260	**.406***	**.548****	**.489****	**.477****
HCY (μM)	**.317***	**.365****	.230	**.268***	.242	**.326***

Abbreviations: HCY, homocysteine; IL-6, interleukin 6.
*P < .05;
**P < .01.
The statistically significant values are reported in bold.

et al.[37] Increased HCY levels may be explained with metabolic alterations related to genetic factors and nutritional issues, which in turn may feature insufficient intake or absorption of vitamin B6, B12, or folate, crucial for the metabolism of HCY.[36,42] Impaired methylation and trans-sulfuration pathways of HCY metabolism may be associated with the reduced DNA methylation, glutathione depletion, and altered redox balance frequently reported among ASD children.[36,42] In addition, increased HCY levels may directly exert a negative impact on these systems.[36,42] In this framework, HCY concentrations could be considered a source of information about redox state and DNA methylation in ASD.[36,42] Among the studies that stressed higher levels of HCY in ASD, some also reported lower values of cysteine, glutathione, and GSH/GSSG ratio, which are considered endogenous antioxidant defenses.[36] Globally, our results support, in an adult sample, the presence of a link between autism spectrum and altered HCY levels, which may be eventually associated with impaired methylation/trans-sulfuration pathways.[36,37]

Noticeably, while both IL-6 and HCY levels have been previously reported to be affected by the presence of anxiety or mood disorders, as well as by psychopharmacological drugs,[4,36,66] we did not find significant differences in the levels of these parameters on the basis of the presence or not of other comorbid conditions or use of pharmacological treatments.[57] This data may lead to hypothesis that in our sample the presence of autism spectrum symptoms overcame the impact on IL-6 and HCY levels of other conditions or treatments.

Correlations between HCY, IL-6 levels, and psychometric instruments

Considering HCY, we found significant correlations with AdAS Spectrum and RAADS-14 total scores, while the highest correlations were reported with the ruminative dimension as measured by the RRS and by the AdAS Spectrum-related domain. However, HCY levels also showed significant correlations with all AdAS Spectrum domains with the exception of *Empathy* and with the RAADS-14 *Mentalizing deficit* and *Social Anxiety* domains. These findings are in line with the correlation between autism symptoms' severity and HCY levels reported in other studies among children.[36] A previous study also stressed a positive correlation between impaired communication abilities and increased HCY levels in children with ASD.[50] Noticeably, other researches in clinical and general populations showed an association of increased HCY and lower folate levels with inflexibility and cognitive impairment.[67,68] Our results may be in accordance with the specific association, reported by other authors, between ruminative thinking and the methylenetetrahydrofolate dehydrogenase 1 like (MTHFD1L) gene allele A polymorphism (*rs11754661*), which is linked to increased levels of HCY, further supporting the possible involvement of HCY metabolism in the pathophysiology of ruminative thinking.[67,68] A biochemical explanation of the link between HCY alteration, cognitive functions, and ruminative thinking may feature different mechanisms that should be investigated in further studies, such as the antagonistic action of HCY on *N*-methyl-ᴅ-aspartate (NMDA), impaired methylation processes, and increase of oxidative stress.[67,68] Ruminative thinking is a feature frequently associated with the autism spectrum, but it is also considered a trans-nosographic dimension, which could be eventually underlain by the presence of autistic traits. The presence of ruminative thinking is typically linked to an increased vulnerability toward the development of psychiatric symptoms, particularly after

stressful events, and with the tendency toward suicidal thoughts and behaviors.[69-72] A partially different pattern was found for the association between IL-6 and psychometric scales. In particular, IL-6 levels did not show significant correlations with the total scores of the AdAS Spectrum and the RAADS-14, showing instead a significant positive correlation with the specific autistic dimension of *Mentalizing deficit* (as measured by the RAADS-14) and with all dimensions of ruminative thinking, as measured by the RRS. Noticeably, IL-6 levels were more strongly correlated with RRS total and domain scores than HCY ones. Starting from the reported role played by IL-6 in different processes of CNS pathophysiology, including in the promotion of sickness behavior,[64] several authors hypothesized a possible IL-6 involvement in the modulation of autistic-like features.[73-75] While in *IL-6/IL-4 knock-out* mouse models cognitive impairment was typically reported, previous literature in ASD children showed an association between communication deficits and cytokine levels, although not specifically IL-6.[4,22,23] IL-6 levels were instead reported to be more linked to other dimensions such as repetitive behaviors,[4,22,23] poorer social relationships,[76,77] and sleep quality.[19,76,78] Somewhat in line with our results, Moriarity et al found that higher levels of rumination were a risk factor for the presence of higher IL-6 concentration, which would lead in turn to symptoms of anxiety and depression.[79] On the other hand, Woody et al reported that neutral reflection seemed instead to be associated with lower IL-6 concentration.[80] Our findings globally support the association between IL-6 and ruminative thinking, although the specific nature of this relationship remains to be clarified: as stated by other authors, while IL-6 may be involved in ruminative thinking pathophysiology through promoting cognitive alterations and depressive mood, the presence of ruminative thinking may be able to promote the enhancement of inflammatory processes, which may feature the increase of IL-6 levels.[76,79] In conclusion, our results suggest that the severity of autistic symptoms as a whole may be associated with increased levels of HCY, while IL-6 concentrations seemed to be more specifically linked to the dimension of ruminative thinking. Finally, considering the impact on work and social functioning, we found that both IL-6 and HCY levels were significantly and positively associated with a greater impairment in work/social adjustment as measured by WSAS total score, being also associated with most of WSAS single domains. However, also in this case the strongest correlations were reported with IL-6 levels. This data seem to suggest that a poorer functional adjustment may be mirrored by a greater impairment also from a biochemical point of view, in particular for immune system alteration. Noticeably, the higher association of IL-6 with both rumination and poorer adjustment reported herein might eventually be in line with the link between ruminative thinking and worse global functioning reported in other studies.[2,54,70-72]

Limits and conclusion

This work should be considered in light of several limits. First of all, the study featured a cross-sectional design, preventing us from making inferences about eventual temporal or causal relationships among the investigated variables. In addition, the small sample size limits the impact and extensibility of our findings. In particular, the limited number of subjects enrolled in the study prevented us from performing stratified analyses in order to evaluate specific correlations within each group, or from correcting our data for age and gender, despite this analysis would have been of great interest for

testing the hypothesis of a continuum in the autism spectrum. Furthermore, although the clinical diagnoses were confirmed by trained psychiatrists, symptoms were evaluated by self-reported psychometric questionnaires, eventually allowing under or over-estimation biases depending on the judgment of the subjects. Despite these limitations, our findings, highlighting a significant increase of IL-6 and HCY levels in adults with ASD, support the hypothesis of a key involvement of HCY-related metabolism and immune system alteration in autism spectrum pathophysiology, as suggested by previous studies among children.[4,36,81] The intermediate levels reported among BAP subjects further highlight the presence of a continuum between subthreshold and full-threshold autism spectrum, which seem to be present also from a biochemical point of view, stressing the importance of a dimensional approach to psychiatric conditions.[8,70,82] Our results also highlight that HCY and IL-6 may possibly show different patterns of association with autism spectrum symptoms, which need to be clarified by further investigations. This study may open the way to future researches, extending a dimensional approach from psychopathology to neurobiology. From a practical and clinical point of view, the identification of biochemical correlates of the autism spectrum, as well as the eventual link between some metabolic alterations and specific clusters of symptoms, may allow for improving diagnostic procedures and also promote the development of new therapeutic targets for this population. On the other hand, increasing our knowledge of biochemical correlates of autism spectrum conditions may shed more light on autism spectrum pathophysiology. Future research in this field should take into account the possible presence of intertwined relationships between different metabolic routes and between peripheral and central systems in shaping neurodevelopmental trajectories. Globally, further studies in wider samples and with a longitudinal design, possibly featuring the use of standardized tools for measuring ASD symptoms (such as the Autism Diagnostic Observation Schedule, ADOS-2) are needed to clarify the association between impaired HCY-related metabolism, immune system alteration and the autism spectrum.

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