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INVESTIGATING BIOCHEMICAL MARKERS OF ADULT AUTISM SPECTRUM DISORDER PHENOTYPES

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Contents

Abstract

Background: Recently, in the framework of a dimensional approach to autism-related psychopathology, increasing attention has been paid on investigating specific features of Autism spectrum disorder (ASD) during adulthood. Particular interest was paid on milder forms of ASD, without intellectual and language impairment, which may remain under-detected in early life. Moreover, several reports highlighted the presence of significant autistic-like traits in non-affected first-relatives of subjects with ASD, leading to the conceptualization of a "Broad autism phenotype" (BAP). Despite an increasing body of data from neuroimaging studies reported neurostructural and neurofunctional alterations in BAP, there is still a lack of evidence about potential biochemical correlates of ASD-like traits. The first attempts in researching biochemical markers of ASD focused on neurotransmitters and in particular on serotonin (5-HT), reporting possible different alterations between adulthood and childhood and/or between different kinds of biological samples. Brain-derived neurotrophic factor (BDNF), tryptophan (TRP), and metabolites of the TRP-derived kynurenine (KYN) shunt were also found altered in ASD children, and authors hypothesized their possible involvement in increased excitotoxicity or inflammatory activity. Other research also reported immune system activation among ASD children: results in this field seem promising, with studies stressing an association of cytokine levels with different grades of clinical severity and specific clusters of symptoms. Moreover, altered homocysteine (HCY) metabolism and altered trans-sulfuration and methylation processes are acquiring growing interest as possible metabolic signatures of ASD. Despite that, for most of these parameters scant research focused on adult samples and/or on BAP, without studies comparing adult ASD patients with their adult relatives. Aims: The aim of this work was to identify possible biochemical correlates for sub-threshold and over-threshold ASD symptomatology in adults. In particular, the study aimed to: evaluate presence and features of ASD symptoms in adult ASD patients without language or intellectual impairment and their first-degree relatives, as well as in controls, through suitable psychometric scales; evaluate levels of various biochemical parameters, among the three groups, and in particular: circulating levels of 5-HT, TRP, KYN, quinolinic acid (QA), chinurenic acid (KYNA), BDNF, IL-6, HCY; evaluate the possible correlations between clinical features, as measured by the psychometric scales, and

biochemical parameters. *Methods:* A sample of adult ASD patients (ASD group) and first-degree relatives of the ASD probands (BAP group) were recruited among patients followed at the Psychiatric Section of Azienda Ospedaliera Universitaria Pisana, whereas unrelated controls (CTL group) were recruited on a voluntary basis. All subjects underwent a psychiatric and biochemical assessment. The psycometric instruments employed were: the Structured Clinical Interview for DSM-5, the Adult Autism Sub-threshold Spectrum (AdAS Spectrum), the Autism-Spectrum Quotient (AQ), the Ritvo Autism Asperger Diagnostic Scale,14-item version (RAADS-14), the Ruminative Response Scale (RRS) as well as the Work and Social Adjustment Scale (WSAS). A sample of peripheral venous blood was withdrawn from all the subjects and then processed for obtaining the different analytical specimen for biochemical assessment: the platelet poor plasma (PPP), platelet pellets and serum. All these parameters were measured by means of dedicated Enzyme-linked immunosorbent assay (ELISA) procedures. *Results*: ASD patients reported significantly higher total scores (greater severity of autistic traits/functional impairment) than the other groups on all psychometric scales. The BAP group reported intermediate scores, significantly higher than the CTL group. At the same time ASD patients reported significantly lower intra-platelet 5-HT, PPP 5-HT and TRP levels than BAP and CTL groups. Significant differences depending on pharmacological treatment within groups were reported for intra-platelet 5-HT levels only, and no difference in intra-platelet 5-HT was found when exluding subjects in treatment with antidepressants from the analysis. Moreover, significantly lower levels of KYNA were reported in both ASD and BAP group when compared with CTL subjects. IL-6 and HCY were instead significantly higher in the ASD group than in the CTL one, with BAP group showing intermediate levels, not significantly different from those reported in the other two groups. A multinomial logistic regression analysis identified higher levels of HCY and IL-6 as the statistically predictive variables of being in the ASD group, while increased IL-6 was statitstically predictive also of being in the BAP group. Specific patterns of association were found between autistic symptoms and biochemical variables. The biochemical parameters most associated with functional impairment were the increased levels of HCY and IL-6. *Conclusions*: our results confirm the need of further research on alterations of TRP metabolism in ASD and highlight the value of assessing the association of ASD with specific immune system alterations and impaired HCY-related metabolism, which may

affect trans-sulfuration/methylation processes in this population. Moreover, our results confirm the presence of intermediate alterations in relatives of ASD patients also from a biochemical point of view, thus providing more support to the presence of a continuum between sub-threshold and full-threshold ASD phenotypes. Finally, our results stress the importance of evaluating metabolomics/proteomic signatures in the field of ASD patients' care and management.

1. Introduction

1.1 Autism spectrum disorder and the Broad autism phenotype

Autism spectrum disorder (ASD) is a condition characterized by an impairment in verbal and non-verbal communication as well as by restricted and repetitive patterns of interests and behaviors. ASD onset takes place in early childhood, causing clinically significant functional impairment (APA 2013). According to the current version, the fifth, of the Diagnostic and Statistical Manual for Mental Disorders (DSM-5), ASD may be or not be associated with intellectual impairment and/or delays in language development, thus including under the single label of "ASD" the previous clinical categories of Autistic disorder (the more severe presentation), Asperger syndrome (which did not feature deficits in cognitive and language development) and Pervasive developmental disorder-not otherwise specified (PDD-NOS) (APA 2013). Among ASD patients, different levels of symptoms severity together with deficits in social and communication skills are detectable, with dramatically variable impacts on general functioning (Dell'Osso et al. 2016; 2017). Notwithstanding research on ASD mainly focused on children, there has recently been a growing interest in evaluating ASD presentations in adulthood, as well as in the possible different features that the disorder may present during different stages of life. In particular, several authors stressed the importance of identifying, among adult populations, milder forms of ASD, without language or intellectual impairment, which often may remain undiagnosed in childhood but which are nevertheless associated with high levels of subjective distress and a limited adjustment to environmental stimuli/stressors (Dell'Osso et al. 2016; 2017).

Since 1977 the important role of genetic heritability in ASD has been highlighted in several studies (Folstein and Rutter 1977; Ronald and Hoekstra 2011). Familiar aggregation is frequently reported in ASD; moreover, it should be noted that often close relatives of ASD subjects, even if not themselves clinically affected by the disorder, show personality traits as well as neurostructural correlates similar to those of their probands, although less severe (Sucksmith et al. 2011; Billeci et al. 2016). These evidences progressively lead to the conceptualization of a "Broad Autism Phenotype" (BAP) (Sucksmith et al. 2011; Dell'Osso et al. 2016; 2017; Carpita et al. 2020a; 2020b), a label used to define the presence of intermediate, sub-threshold phenotypes in the autism spectrum. This condition features ASD-like traits such as narrow interests,

repetitive behaviors, but also deficits in social communications and social skills (Baron-Cohen and Hammer 1997; Sucksmith et al. 2011; Billeci et al. 2016). BAP prevalence is higher among close relatives of ASD patients than among general population (Bailey et al. 1998; Losh et al. 2008), but it can be found also in some high-risk groups (Dell'Osso et al. 2017), and it is associated with a higher vulnerability towards the development of other psychiatric disorders as well as suicidal ideation and behaviors (Dell'Osso et al. 2017; Carpita et al. 2020b). In this framework, an increasing body of evidences from neuroimaging corroborates the presence of significant neurostructural and neurofunctional alterations in BAP (Billeci et al. 2016; Brondino et al. 2018; Carpita et al. 2020b). However, there is still a lack of biochemical research on this specific matter (Brondino et al. 2018; Carpita et al. 2020a).

A better understanding of biochemical correlates of autism spectrum conditions may led to an improvement in our knowledge about ASD pathophysiological mechanisms. Moreover, the specific need of identifying biochemical markers in ASD in order to improve diagnostic procedures and treatment options has been previously stressed in literature, given the extremely heterogeneous presentation of the disorder (Gabriele et al. 2014).

1.2 The link between autism spectrum and monoamines: focus on serotonin

The first attempts in researching biochemical markers of ASD focused on neurotransmitters, and in particular on serotonin (5-hydroxytryptamine, 5-HT) (Schain and Freedman 1961). Some interest has been paid to dopamine, due to the crucial role of dopaminergic system in ASD, as demonstrated by animal models and genetic studies, as well as by the clinical efficacy of antipsychotics targeting the D2 receptors in treating some symptoms of these patients (Lam et al. 2006; Eissa et al. 2014; Pavăl 2017). Thus, a more limited number of studies have also evaluated levels of dopamine and/or of its metabolite homovanillic acid in blood, urine or cerebrospinal fluid (CSF), showing higher values of these analytes in ASD patients, a finding so far deemed as controversial (Lam et al. 2006; Elissa et al. 2014). A small number of studies also addressed norepinephrine and its metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG) circulating concentrations, reporting higher levels in ASD children (Lam et al. 2006;

Żurawicz et al. 2013). However, the most interesting and replicable result in the field of monoaminergic alterations related to ASD was the report of increased levels of 5-HT in ASD children. The link between ASD and 5-HT was supported also by studies focusing on the 5-HT transporter (SERT) as well as by genetic studies and neuroimaging studies (Lam et al. 2006; Eissa et al. 2014; Gabriele et al. 2014). To date, the presence of high blood levels of 5-HT is still one of the traits most consistently associated with ASD, as highlighted by a wide number of researches, although only a few of them focused on adult samples (Gabriele et al. 2014). Some authors focused also on the issue of BAP investigating 5-HT levels in ASD probands and in their first-degree relatives, but these studies usually featured child probands and/or did not include unrelated controls, an aspect which needs instead to be addressed as already stressed in current literature (Gabriele et al. 2014).

5-HT has a pervasive role in the body. Its functions range from regulating a wide set of human behaviors in the central nervous system (CNS) to regulating motor output, blood vessels dilatation, several gastrointestinal functions and the immune response. For this reason, its role in other psychiatric disorders has been widely investigated, in particular in the field of mood and anxiety disorders. It also should be remembered that several psychiatric drugs, from selective 5-HT reuptake inhibitors (SSRIs) to atypical antipsychotics, target the 5-HT system. 5-HT receptors are ubiquitous in the body, featuring a wide heterogeneity of subtypes, at least 14 different ones (Harrington et al. 2013; Muller et al. 2016).

As other main monoamine neurotransmitters, 5-HT is produced from an essential amino acid, in this case tryptophan (TRP), which cannot be synthesized by the human body and must be introduced through the diet. During the process, TRP is converted to 5-hydroxyTRP (5-HTP) by the enzyme tryptophan hydroxylase (TPH), which is available in two isoforms: one is active in the periphery or in the pineal gland (TPH1), while the other (TPH2) works in the CNS. Finally, the ubiquitous aromatic acid decarboxylase (AADC) converts 5-HTP in 5-HT (Harrington et al. 2013).

The main responsible for 5-HT degradation is the monoamine oxidase A (MAOA), with the resulting production of 5-hydroxyindoleacetic acid (5-HIAA) (Muller et al. 2016). 5-HT is also used as a substrate for the synthesis of melatonin, a well-known compound endowed with a crucial role in regulating sleep-wake cycles depending on daily light and photoperiod. Neuronal 5-HT is stored into pre-synaptic vesicles and released in the

synaptic cleft after neuronal depolarization. Subsequently, 5-HT may be uptaken again by SERT (the 5-HT reuptake system) inside neurons, where it is restored inside vesicles or MAO-degraded (Harrington et al. 2013; Muller et al. 2016). 5-HT auto-receptors, located in the presynaptic cells, exert a crucial role in modulating 5-HT homeostasis through feedback mechanisms (Muller et al. 2016). An overview on the 5-HT system is provided in **figure I1**.

Noticeably, although 5-HT is synthetized in both CNS (at the level of raphe nuclei, which project to several other brain regions) and in the periphery (mainly in the gut by enterochromaffin cells), it cannot cross the blood brain barrier (BBB). The quantity of 5-HT produced in the periphery greatly overcomes that produced in the CNS. Most of peripheral 5-HT is stored in enterochromaffin cells, while a smaller quantity pass in the bloodstream, where the 99 % is up-taken by SERT and stored in platelets. However, this large amount of 5-HT does not directly interact with the neural tissue of the CNS (Harrington et al. 2013). In this latter, serotonergic neurons located in the raphe nuclei innervate a wide number of brain regions, and their effects play a crucial role in modulating human behavior (Muller et al. 2016). Several researches pointed out the potential impact of 5-HT on neurodevelopment. The production of 5-HT in the periphery dramatically increases during pregnancy, when this monoamine is also produced by the placenta, while the embryo is able to autonomously produce 5-HT after the fifth week of gestation.

It seems that 5-HT could be involved in the modulation of neurodevelopment, including the development of dopamine system, influencing neuronal maturation, neural migration and synaptogenesis. Several rat models showed the disruptive effect of decreased or increased 5-HT levels during development (Harrington et al. 2013). After birth, 5-HT levels are at their highest during the first years of life, progressively decreasing to adult levels around the fifth year (Muller et al. 2016). The specific role of SERT was also investigated, pointing out its crucial impact in neurodevelopment and its role in psychiatric disorders, with genetic SERT variations moderating the risk of developing psychiatric symptoms after life events (Caspi et al. 2003; Harrington et al. 2013).

In this framework, the finding of high 5-HT levels in platelets among autistic children increased, during the early ’60, the interest in investigating the role of 5-HT in ASD.

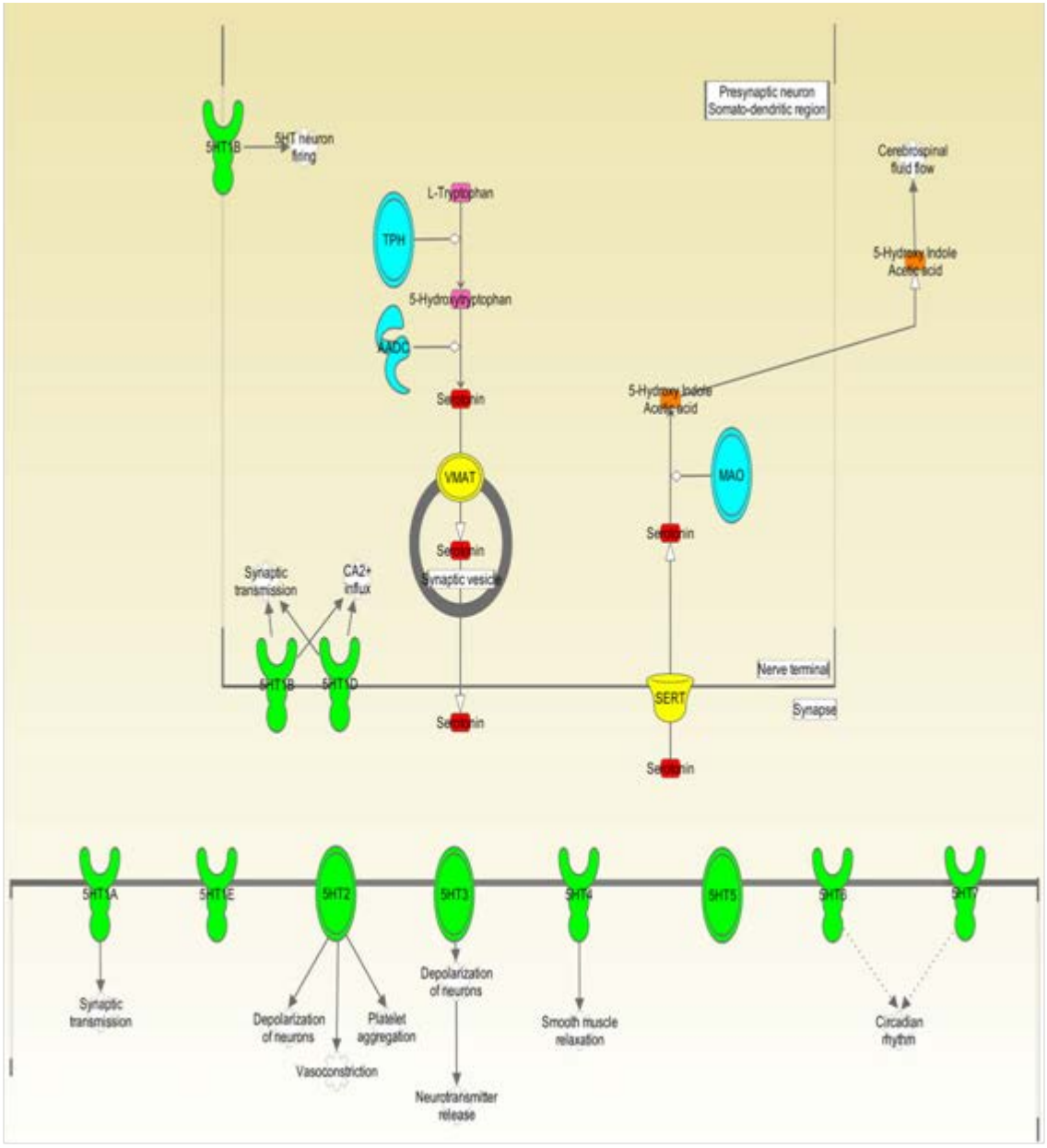

Figure I1. 5-HT synthesis, release, reuptake and degradation mechanisms, with principal functions of 5-HT receptors. AADC: aromatic acid decarboxylase; MAO: monoamine oxidase; VMAT: vesicular monoamine transporter. SERT: serotonin transporter; 5HT1-7: 5-HT receptor sub-groups are reported in oval shape, individual receptors in goblet shape (from Muller et al. 2016).

However, it should be noted that, as reported in the first studies in this field and subsequently confirmed by others, only a sub-group of subjects with ASD, estimated

around 30 %, actually showed hyperserotonemia in platelets. The underlying pathophysiological mechanisms of hyperserotonemia in ASD, and only in a sub-group of ASD subjects in particular, are still debated (Mulder et al. 2004; Muller et al. 2016). According to some authors, hyperserotonemia in ASD populations may be linked to specific behaviors such as stereotypies and self-injuring, but not all the studies confirmed these findings (Kolevson et al. 2010; Sacco et al. 2010; Muller et al. 2016). On the other hand, scant literature focused on the relationship between 5-HT levels and other kinds of ASD symptoms, and, even more importantly, on the link between central and peripheral levels of 5-HT in ASD (Hranilovic et al. 2009). A possible association with hyperserotonemia and more severe gastrointestinal symptoms among children with ASD has also been suggested (Muller et al. 2016).

As reported above, 5-HT cannot pass through the BBB, and, as a consequence, peripheral 5-HT levels do not necessarily give information about 5-HT levels in the CNS. Some researchers also suggested that CNS levels of 5-HT may be lower in ASD patients. However, studies on CSF led to controversial results: some authors did not find significant differences in 5-HIAA levels among children with or without ASD (Narayan et al. 1993; Harrington et al. 2013), while neuroimaging studies reported altered 5-HT synthesis in ASD children depending on the brain region. Other authors instead stressed an altered pattern of 5-HT synthesis in ASD, and in particular a reduction in the first years of development and an increase after the fifth year (Chugani et al. 1997; 1999; Harrington et al. 2013; Muller et al. 2016).

Intriguingly, serotonergic drugs seem to improve some ASD symptoms, and in particular SSRIs were reported to reduce repetitive behaviors and improve social and global functioning in ASD adults (Hollander et al. 2012; Harrington et al. 2013). Results from the studies on beneficial effects of SSRIs among ASD children or adolescents led to contrasting results, possibly also due to a lack of homogeneity in methodology and an increased incidence of side effects (Williams et al. 2010; Muller et al. 2013). It should be noted that the currently approved drugs for ASD in children, risperidone and aripiprazole, exert their effects on different systems of monoamines, including 5-HT (Muller et al. 2016).

The mechanisms involved in higher levels of 5-HT in ASD may range from a decreased catabolism, a higher production by enterochromaffin cells or an alteration of the uptake into platelets (Harrington et al. 2013; Muller et al. 2016). While possible alterations of

5-HT gut production in ASD remain under-investigated, a wide number of studies focused on platelets. In platelets, three proteins are tightly associated with 5-HT physiology: the SERT, the degradation enzyme MAOB and the 5-HT receptors (specifically, the 5-HT2A) (Hranilovic et al. 2009). Most of the studies focused on the SERT. A possible link between 5-HT uptake velocity and whole blood 5-HT levels was reported in both ASD subjects and their relatives (Anderson et al. 2002; Muller et al. 2016). However, no specific changes in SERT binding were found, and no difference was reported between ASD subjects with or without high levels of 5-HT with respect to 5-HT uptake (Anderson et al. 2002; Muller et al. 2016).

Neuroimaging studies reported a decreased SERT binding in ASD children and young adults, but a study in high-functioning adults did not replicate this result (Nakamura et al. 2010; Girgis et al. 2011; Muller et al. 2016). 5-HT receptor binding was instead found decreased among ASD subjects with high levels of 5-HT and also among their relatives, although this result was not unanimously confirmed (Perry et al. 1991; Cook et al. 1993; Muller et al. 2016).

Hranilovic et al. (2007) evaluated a sample of ASD adults, identifying a sub-group with high platelet 5-HT levels, without finding a relationship between 5-HT levels and symptom severity or intellectual disability; only a correlation between 5-HT amounts and speech development was reported. In a further study, they reported a higher mean velocity of MAOB kinetics in ASD subjects than in controls, with the greatest levels reported by hyperserotonemic ASD subjects. However, they did not find any difference with respect to SERT kinetics (Hranilovic et al. 2009). Considering that 5-HT-associated proteins expressed in platelets are encoded by the same genes of their CNS counterparts, these authors suggested that hyperserotonemia in the periphery might be considered a marker of a 5-HT system alteration also into the CNS, which would result in the developmental impairment underlying ASD symptoms. On the other hand, higher peripheral 5-HT levels may exert a role in ASD-related brain alteration during intrauterine life, before the development of the BBB (Hranilovic et al. 2009).

In this framework, some researchers highlighted a reduced 5-HT2A receptor binding among high-functioning ASD adults or in parents of ASD children with neuroimaging (Murphy et al. 2006; Goldberg et al. 2009), pointing out that altered 5-HT levels in the periphery may be also a marker of an altered 5-HT system in the brain (Muller et al. 2016).

Another factor implied in 5-HT system regulation is oxytocin (OT), a nonapeptide related to social behaviors, whose receptors were reported to be expressed in 5-HT cells of the CNS, thus influencing 5-HT release and 5HT receptor availability (Dolen et al. 2013; Yoshida et al. 2009; Muller et al. 2016). Notwithstanding studies which investigated OT itself as a biomarker for ASD did not report consistent results, some studies reported an association between ASD symptoms and the interaction between 5-HT- and OT-related gene polymorphisms (Nyffeler et al. 2014). Among ASD children and adolescents, peripheral 5-HT levels, measured in the whole blood, seem to show a negative correlation with plasma OT levels, while higher 5-HT levels were observed in a *knockout* mouse model lacking OT receptors (Hammock et al. 2012; Muller et al. 2016).

Several authors investigated the link between 5-HT and autism by means of a genetic approach. The human gene encoding SERT in both the CNS and the periphery, named *SLC6A4*, was considered one of the potential genes related to ASD, and as such has been the focus of several investigations. One of the most interesting findings in this field was the reported association of ASD with the short variant of the SERT-linked polymorphic region (*5-HTTLPR*), a polymorphic region of *SLC6A4* related to a reduced functionality and expression of this gene (Harrington et al. 2013). Moreover, it seems that subjects with the short variant would show a higher risk towards developing mood symptoms after a traumatic experience (Caspi et al. 2003; Harrington et al. 2013). However, it should be noted that the literature displays an extreme variability in the results: not all the studies confirmed the association between ASD and the short variant of *5-HTTLPR*, while some authors conversely reported an over transmission of the long variant among subjects with ASD or did not find any association with *5-HTTLPR* variants (Devlin et al. 2005; Ramoz et al. 2006; Tassone et al. 2011; Harrington et al. 2013). Other studies also highlighted, among ASD children, an association between the *5-HTTLPR* short allele and an increased cortical grey matter volume, another frequently established feature of ASD in childhood (Wassink et al. 2007; Muller et al. 2016).

A high variability in the results characterizes also the research on the association between ASD and *MAOA* gene polymorphisms: studies alternatively found an association between ASD and the variant linked to increased or decreased gene transcription. Some authors hypothesized an involvement of the maternal phenotype

and/or the parent-of-origin effects of the allele, due to the X chromosome location of this gene (Tassone et al. 2011; Harrington et al. 2013).

Although hyperserotonemia showed a high heritability, it should be noted that most of the studies on *SLC6A4*, one of the most well-known genes in this field, did not concomitantly evaluate 5-HT blood levels (Muller et al. 2016). Some studies reported an association between *SLC6A4* variants and 5-HT levels, although with controversial results (Weiss et al. 2005; Cross et al. 2008; Muller et al. 2016), whereas others highlighted an association between 5-HT levels, the polymorphism of the integrin β3 (ITGB3) gene and vitamin D receptor, which seems involved in the regulation of 5-HT synthesis (Weiss et al. 2004; Muller et al. 2016). ITGB3 is a protein that influences the function of SERT: mice lacking ITGB3 showed repetitive behavioral traits and social cognition impairment (Carter et al. 2011; Muller et al. 2016). Some authors also highlighted the possibility that the interaction of *SLC6A4* and *ITGB3* gene polymorphisms, together with other factors, may influence ASD risk and the 5-HT system in a broader way (Cross et al. 2008; Muller et al. 2016).

Several mouse models were employed in this field. Studying ASD genetic mouse models, such as the inbred BTBR mouse strain, which is characterized by repetitive behaviors and impaired social interaction and communication, some studies reported the beneficial effect of TRP supplements or of fluoxetine, an SSRI drug, on sociability features (Meyza et al. 2013; Gould et al. 2014). An increased 5-HT1A activity in the hippocampus and a reduced SERT binding in the whole CNS were also reported in BTBR mice (Gould et al. 2014; Zhang et al. 2015; Muller et al. 2016). Limited literature investigated the association with blood 5-HT levels. *SERT* or *MAOA knockout* mice were reported to show autistic- or anxiety-like behaviors together with an altered CNS architecture in several brain regions; however, these changes were associated with decreased platelet 5-HT levels (Murphy and Lesh 2008; Muller et al. 2016). On the other hand, mouse models lacking 5-HT1A receptors showed an increase of 5-HT levels in the second week after birth (Janusonis et al. 2006; Muller et al. 2016). The *Ala56* allele is one of the most frequent *SERT* gene variant: *Ala56* mouse genetic models, displaying an altered SERT function, have shown higher whole blood 5-HT levels, accompanied by repetitive behaviors and altered social functions (Veenstra-VanderWeele et al. 2012; Muller et al. 2016). Other studies featured *knockout* mouse

models for other 5-HT system proteins, such as TPH2, finding associations with altered social cognition and inflexibility (Mosienko et al. 2015; Muller et al. 2016).

As reported above, despite 5-HT system is one of the most investigated topics in the field of ASD, it is still not known why hyperserotonemia is detectable in only about a third of ASD subjects, and if this biological phenotype should be considered an endophenotype or if it is rather connected to some symptoms' clusters, to severity levels or to specific ASD presentations (Hranilovic et al. 2007; 2009).

Gabriele et al. (2014) performed a meta-analysis on twenty-two previous studies, not controlling for age and ethnicity but distinguishing previous researches depending on the different biomaterials and analytical procedures employed. Regarding analytical method, the authors did not find significant differences between results obtained from studies performed by High Performance Liquid Chromatography (HPLC) and those performed by fluorometric assays (both reporting similar mean odd ratios) (Gabriele et al. 2014). For measuring hyperserotonemia, accordingly to previous studies (Hranilovic et al. 2007), these authors considered the mean 5-HT value reported in the control group + 2 Standard deviation (SD) as the cut-off upper limit of the normal distribution (Gabriele et al. 2014). Considering whole blood, they found 15 studies, reporting in the meta-analytic results a global mean percentage of hyperserotonemic ASD subjects that accounted for about 28 % of the whole sample, with higher levels of 5-HT among ASD subjects than among controls (Gabriele et al. 2014). However, the authors highlighted a great heterogeneity of results in the considered studies, which was apparently independent from the normalization or not of 5-HT levels by platelet numbers. Moreover, they identified 3 studies, performed on platelet-poor plasma (PPP), for which no significant difference between patients and controls was reported in the meta-analysis. The authors reported also 6 publications where 5-HT levels were measured in the platelet-rich plasma (PRP), which highlighted significant differences between ASD and control subjects, with a global rate of hyperserotonemia accounting for about 25 % of the sample (Gabriele et al. 2014). However, only 4 of these studies reported 5-HT concentrations normalized by platelet count. A more recent review pointed out that not only studies on PPP, but also studies on smaller samples might did not find differences in 5-HT levels between ASD and control subjects, due to the specific distribution of hyperserotonemia in ASD (Hranilovic et al. 2007; Padmakumar et al. 2019). These authors further highlighted the impact of age on 5-HT levels, which are often higher in

studies on prepuberal ASD children (Padmakumar et al. 2019). In this framework, some authors also questioned if higher 5-HT levels should be considered a trait typical only of ASD children or it may extend to adulthood, considering that most of the available literature focused on ASD during childhood; in addition, most of the limited number of studies featuring ASD adults or ASD young adults were conducted in small samples or in mixed samples of subjects with different age (Hranilovic et al. 2009; Gabriele et al. 2014). Moreover, the few studies focused only on ASD adults usually included subjects of relatively young age (Gabriele et al. 2014).

One of the first studies featuring ASD adults was led by Minderaa et al. (1989), who enrolled 40 students with an ASD diagnosis with a mean age of 19.4±4.9 years and 20 controls (mean age = 22.0±7.5 years), reporting significantly higher whole blood 5-HT levels among unmedicated ASD subjects (n = 17) than in controls, while medicated ASD subjects reported significantly lower 5-HT levels than the unmedicated ones. Piven et al. (1991), reported that ASD subjects with other relatives affected by Pervasive developmental disorders (PDD) (n = 23) showed higher PRP 5-HT levels than ASD subjects (n = 5) without affected relatives, while both groups showed higher 5-HT levels than controls (n = 10). It should be noted that, although this study also included ASD adult subjects, the authors enrolled subjects of different age in the same groups, with an age range varying between 6 and 53 years for the ASD group and an age range between 4 and 47 years for the control group. More recently, Hranilovic et al. (2007), in a sample of ASD subjects (n = 63, mean age = 26.1±6.6 years), found a 32 % of hyperserotonemia and higher platelet 5-HT levels when compared with a control group (mean age = 39.9±9.2 years). However, several subjects in the sample were medicated. These authors reported a significant effect of SSRIs on intra-platelet 5-HT levels, while no effect was found for the other drugs: subsequently, they removed subjects treated with SSRI drugs before performing the comparison. In addition, 5-HT levels did not seem to be related with the severity of autism symptoms or with the degree of mental retardation, but they were significantly correlated with verbal communication impairment and speech development alteration (Hranilovic et al. 2007). On the other hand, previous studies on adult samples did not find significant differences between young adults with ASD and controls with respect to 5-HT levels: McBride et al. (1998) reported significantly lower whole blood 5-HT levels in controls when compared with prepuberal ASD subjects but not when compared with post-puberal ASD

ones. Similar results were obtained by Croonenbergs et al. (2000), who failed to find differences in platelet 5-HT levels between ASD adolescents (12-18 years) and controls. Hranilovic et al. (2007) stated that these controversial results may be due the smaller samples size (< 25 subjects) of the above reported studies, or, conversely, to the higher grade of intellectual impairment in his own study sample. They also hypothesized that hyperserotonemia, although eventually present, might actually be more unusual as a marker of ASD in adults than in childhood. In a wider but younger sample of ASD subjects (n = 81; mean age = 12.57±3.5 years), Mulder et al. (2004) reported instead a 25 % rate of hyperserotonemia in the patient group. On the other hand, Vered et al. (2003) showed significantly lower levels of 5-HT among young adults with ASD (mean age = 25.4±4.8 years) than in controls, although 5-HT levels were measured in PPP. Spivak et al. (2004) also reported reduced 5-HT levels in the PPP among ASD adults (mean age 24.37±4.5 years), highlighting a negative correlation of 5-HT with aggressive behaviors. More recent studies similarly lead to contrasting results. Pagan et al. (2014) reported a rate of hyperserotonemia (defined as a value above 95th percentile of the control group) around 40 % in a sample of 278 ASD patients, without significant differences between patients aged below 16 years and patients aged 16 years or older. On the other hand, Shuffrey et al. (2017), in a wide sample of 292 ASD subjects, observed higher whole blood 5-HT levels in prepuberal ASD patients (mean age = 7.56±2.41 years) than in post-puberal ones (mean age = 16.72±4.72 years), as well as higher hyperserotonemia state (42 % vs. 24 %), although the authors did not include a control group in this study. They also highlighted, only in the prepuberal group, a higher tendency towards hyperserotonemia in males than females.

In summary, results in adult samples seem to show that the prevalence and significance of higher 5-HT among ASD adults remain to be further clarified, keeping in mind that in this population hyperserotonemia might be a lesser stable marker than in younger subjects (Hranilovic et al. 2007; Gabriele et al. 2014).

The involvement of relatives of ASD patients in studies focused on 5-HT levels was managed by heterogeneous protocols: some studies featured the relatives as an intermediate group, while others featured them as a control group (Gabriele et al. 2014). Despite this issue, available studies on this topic reported intermediately higher levels of 5-HT in this population. Kuperman et al. (1985), in a study involving ASD children and their first-degree relatives, reported that ASD boys showed higher PRP and whole blood

levels of 5-HT when compared with ASD girls and with the father group, but not when compared with the mother or the sibling groups. Leventhal et al. (1990), by evaluating 47 families of ASD probands, reported that around a half of the families had at least one hyperserotonemic member. These authors also reported a risk 2.4 times higher of showing hyperserotonemia among relatives of a hyperserotonemic ASD child, although whole blood mean 5-HT levels remained higher in the ASD group than in the relative one. Cook et al. (1990) reported that whole blood 5-HT levels were negatively correlated with vocabulary performance in a group of ASD children (n = 16), and, by evaluating parents and siblings of ASD probands, they also found that the majority of families with at least one hyperserotonemic subject had other hyperserotonemic members, further stressing the familiarity of blood 5-HT levels. Leboyer et al. (1999) highlighted increased levels of 5-HT in parents and siblings (n = 122) of ASD probands (n = 62) when compared with controls. In particular, ASD probands and all the relative groups showed significantly higher 5-HT levels than controls aged > 16 years, but no significant difference was found when comparing the same groups with younger controls. Siblings also showed significantly higher 5-HT levels than their parents. Pagan et al. (2014), when comparing ASD subjects of different age (n = 278) with their unaffected parents or siblings (n = 377) and controls (n = 416), reported that both the patient and the relative groups had significantly higher rates of whole blood hyperserotonemia than controls, ranging between 17 and 10 % for the relative group (higher values were present among mothers) and around 40 % for the ASD group. More recently, Bijl et al. (2015) reported significantly higher PPP 5-HT levels among ASD subjects (n = 30, mean age = 11.9±3.8 years) and their siblings (n = 30) than among age-matched controls (n = 15). It should be noted that some of these authors also pointed out an increased platelet count in ASD patients and their relatives (Bijl et al. 2015). Intriguingly, a study by Connors et al. (2006), highlighted significantly lower PPP 5-HT levels among mothers of ASD subjects (n = 17) than among mothers of unaffected children (n = 8), as well as a significantly lower 5-HT concentrations in ASD children (n = 17, age range = 2-18 years) and their mothers than in their fathers (n = 12) and siblings (n = 7). The authors hypothesized a possible impact of low maternal plasma 5-HT levels for fetal brain development.

1.3 Autism spectrum and TRP metabolism: the kynurenine pathway

TRP is an essential amino acid and a precursor of 5-HT (see paragraph **1.2**). This implies that diet composition can have an impact on TRP endogenous levels and, consequently, on its availability to metabolism (Palego et al. 2016; Badawy 2017). High TRP amounts may be found in several foods, from meat and fish to cereals, milk, chocolate and bananas (Kałużna-Czaplińska et al. 2017). As other large neutral amino acids (LNAAs), TRP can pass the BBB through a competitive transport carrier, suggesting that peripheral TRP levels or the ratio TRP/LNAAs (LNAAs = sum of Phenylalanine, Tyrosine, Valine, Leucine and Isoleucine) may also reflect the availability of this amino acid for 5-HT and melatonin synthesis in the CNS, for which TRP is the principal source (Kałużna-Czaplińska et al. 2017; Savino et al. 2020). In the field of ASD related studies, the interest concerning TRP metabolism arises not only from its link to 5-HT transmission, but also from its possible impact on neuroinflammation through affetting the balance between excitatory and inhibitory processes (Lim et al. 2016). An impaired balance between glutamate-linked excitatory transmission and gamma-aminobutyric acid (GABA) inhibitory action may indeed generate excitotoxic effects (Rubenstein and Merzenich 2003; Lim et al. 2016). Neuroimaging studies also stressed the presence of increased glutamate and glutamine levels in the hippocampus, amygdala and auditory cortex of ASD patients (Brown et al. 2013; Lim et al. 2016). In this framework, it should be noted that the relevance of TRP metabolism is not limited to the 5-HT pathway: in fact, other TRP-derived metabolites play a crucial role in many additional processes, such as the immune and inflammatory responses, oxidative stress regulation, being key mediators between the immune and neuroendocrine systems (Kałużna-Czaplińska et al. 2017; Savino et al. 2020). The amount of TRP metabolized into 5-HT through the methoxyindole pathway is about only 1-2 % of the total one, since the main biotransformation of this essential amino acid is represented by the kynurenine (KYN) shunt. It is supposed that only the 0.5 % of total TRP in the body would be escreted unchanged (Kałużna-Czaplińska et al. 2017). The KYN pathway, also known as the TRP catabolite (TRYCAT) pathway, leads to the formation of several crucial compounds, including nicotinamide adenine dinucleotide (NAD^+) and adenosine triphosphate (ATP). Firstly, TRP is converted to N-formyl KYN

by the indole-2,3-dioxygenase (IDO) or, in a minor quote and mainly in the liver and kidneys, by the TRP-2,3-dioxygenase (TDO). Subsequently, the KYN formamidase metabolizes N-formyl KYN into L-KYN. After that, the pathway further splits in different possible ways. One way is known as the "neurotoxic branch": starting from KYN, it can lead to the production of quinolinic acid (QUIN), or also of picolinic acid (PA), through the production of 3-hydroxy-L-KYN (3-HK) and 3-hydroxyanthranilic acid (3-HAA). Moreover, QUIN can be further transformed, by QUIN phosphoribosyl transferase (QPRT), into NAD^+. The other path originating from KYN is the "neuroprotective branch", through the transformation of this metabolite into kynurenic acid (KYNA) by the enzyme KYN aminotransferase (KAT) (Bilgiç et al. 2020; Savino et al. 2020) (see **figure I2**).

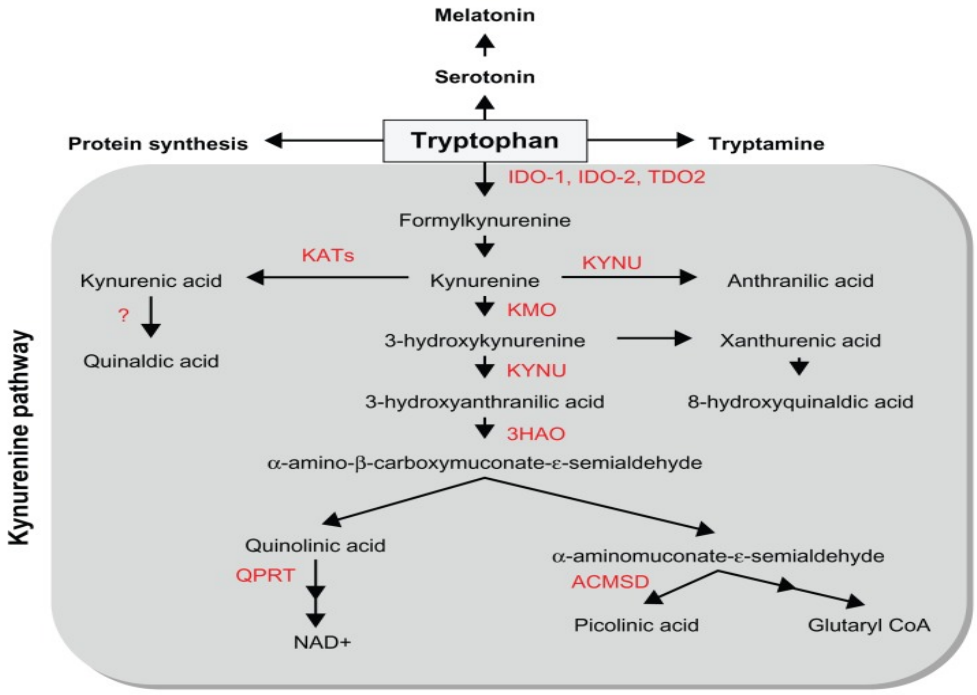

Figure I2. Schematic representation of the KYN pathway. ACMSD: Aminocarboxymuconate-semialdehyde decarboxylase; IDO: Indoleamine 2,3-dioxygenase; KYNU: Kynureninase; KATs: Kynurenine aminotransferases; KMO: Kynurenine 3-monooxygenase; QPRT: quinolinic acid phosphoribosyltransferase; TDO2: Tryptophan 2,3-dioxygenase; 3HAO: 3-hydroxyanthranilic acid oxygenase. (from Jones et al. 2013).

Noticeably, KYNA, 3-HAA and QA cannot cross the BBB, through which TRP and KYN can be instead transported by the LNAA transporter system (Bryn et al. 2017). In the CNS, while KYNA is known to exert a neuroprotective effect, the QUIN pathway has been linked to potentially neurotoxic effects. In particular, KYNA is an antagonist of N-methyl-D-aspartate (NMDA) receptors, and eventually modulates excitotoxic processes linked to glutamate transmission (Bilgiç et al. 2020; Savino et al. 2020). KYNA is also an antagonist of kainate and α-7 nicotinic acetyl choline receptors, whereas QUIN is instead an agonist of them (Savino et al. 2020; Bilgiç et al. 2020). It should be also pointed out that these receptors were reported to be more expressed during the brain development, with the cholinergic transmission playing a fundamental role in the modulation of neurodevelopment, synaptogenesis and neural differentiation (Yakel 2014; Savino et al. 2020). Both KYNA and QUIN are considered relevant modulatory compounds of neuronal circuit modelling in specific brain areas during the development of CNS (Notarangelo and Pocivavsek 2017), while genetic, transcriptomic and proteomic studies highlighted an involvement in ASD pathogenesis of genes, RNAs and proteins that display a function in synapses and synaptogenesis (Bourgeron 2016; Kichukova et al. 2017; Pintacuda et al. 2021). KYNA levels have also been found increased during intrauterine life, rapidly decreasing after birth, possibly to allow the disinhibition of NMDA transmission, which would be a necessary condition for brain development at that stage of life (Notarangelo and Pocivavsek 2017; Savino et al. 2020). Other potentially protective functions of KYNA are represented by its role in inducing the amyloid degrading enzyme, in scavenging free-radicals and in acting as an antioxidant. Moreover, KYNA showed an anti-inflammatory action as an agonist of G-protein coupled receptor (GPR35). Through this latter, it inhibits in the neural tissues the N-type Ca channels and regulates the production of cyclic adenosine monophosphate (c-AMP) (Bilgiç et al. 2020; Savino et al. 2020). KYNA seems to exert an agonistic activity also on the aryl hydrocarbon receptor (AhR), which, among its functions, is reputed to interrupt the release of cytokines in macrophages and other kinds of cells (Savino et al. 2020). Through this latter, KYNA inhibits the N-type Ca channels in neural tissues and regulates the production of c-AMP (Bilgiç et al. 2020; Savino et al. 2020). KYNA seems to exert an agonistic activity also on the AhR, which, among its functions, is supposed to interrupt the release of cytokines in macrophages and other kinds of cells (Zhao et al. 2014; Savino et al. 2020). On the other hand, if

decreased levels of KYNA may reduce the neuroprotective potential in the CNS, also increased levels may exert a detrimental effect: as an example, an over-reduction of the cholinergic transmission (which was found altered in several disorders, such as Alzheimer disease, epilepsy and Schizophrenia) was linked to cognitive deficits and, intriguingly, to specific deficits in attention, memory and learning abilities, which are often impaired in ASD (Deutsch et al. 2010; Savino et al. 2020). The inhibition of NMDA and cholinergic receptors caused by increased KYNA levels seems to lead, in rats, to the disruption of auditory sensory gating. This latter was also highlighted in the rat models of autism, being associated with altered sensitivity to stimuli (Bilgiç et al. 2020).

Conversely, QUIN seems to activate NMDA receptors. It has been hypothesized that the physiological role of this molecule is linked to its action as a glutamatergic excitotoxin produced in response to inflammation, in order to provide more energy to cells by increasing NAD^+ levels: intriguingly, NAD^+ levels have been found reduced in ASD children (Essa et al. 2013; Savino et al. 2020). QUIN accumulation may result from the saturation, by high QUIN concentrations, of the QPRT enzyme, by which QUIN is transformed into NAD^+ (Savino et al. 2020). QUIN is also involved in free radicals and oxidative stress generation, including the increase of pro-oxidant oxygen atoms/molecules such as reactive oxygen species (ROS) with consequent lipid peroxidation, together with the reduction of the antioxidant power, such as decreased glutathione levels and Superoxide dismutase (SOD) activity (Savino et al. 2020). In addition, QUIN is involved in the elevation of the levels of intracellular calcium and of neuronal activity, leading to an impairment in cell homeostasis and mitochondrial functions, eventually inducing apoptosis (Williams et al. 2017; Savino et al. 2020). QUIN seems to act selectively on specific types of NMDA receptors, distributed in brain regions such as the striatum and the hippocampus (Lugo-Huitrón R et al. 2013; Savino et al. 2020). Its neurotoxic actions have been reported in inflammatory CNS diseases, and its elevation may lead to an impaired activation state of NMDA receptors. Among ASD children, an increased glutamate concentration has been reported in the amygdala and hippocampus (Savino et al. 2020). It was thus hypothesized that the excitotoxicity related to QUIN activity may eventually have a role in ASD, presumably by altering neuronal development, formation of synapses and neuronal connectivity. The damaging effect might take place as soon as during intrauterine life (Savino et al.

2020). In line with this hypothesis, some authors pointed out the beneficial effects of NMDA antagonists (and specifically memantine) on ASD patients (Ghaleiha et al. 2013; Savino et al. 2020).

Other metabolites of the KYN neurotoxic branch may also have detrimental effects, as in the case of the induction of oxidative stress by 3-HK and 3-HAA and of the induction of apoptosis by 3-HK (Bryn et al. 2017; Bilgiç et al. 2020). On the other hand, 3-HAA may also suppress the immune and inflammatory activity, acting as a neuroprotective agent.

It should be noted that, in turn, inflammatory processes may induce, in a vicious circle, the TRP metabolism through the KYN pathway: several cytokines, such as interleukine (IL) IL-6, IL-1β or Tumor necrosis factor-α (TNF-α) may activate the limiting enzymes of the KYN pathway, such as IDO and TDO activities (see **figure I3**). Globally, it was hypothesized that the presence of an enhanced inflammatory activity in ASD may activate also in microglia the key enzymes of KYN pathway, such as IDO and KYN 3-monooxygenase (KMO), altering the regulation of this route but also leading to a depletion of 5-HT and melatonin (Savino et al. 2020). The inflammatory induction of KYN pathway may have a role also in the intrauterine-life, eventually impairing the development of the thalamo-cortical fibers mediated by 5-HT. It has been reported that maternal inflammation was associated to an increased TRP metabolism through the KYN pathway, with a reduction of TRP metabolism through the methoxyindole route in rabbit models (Williams et al. 2017; Savino et al. 2020). Adrenal cortisol hormones, whose secretion is increased by psychological distress, can also induce the KYN pathway, specifically shifting it towards the production of QUIN (Tordjman et al. 2014; Savino et al. 2020). Moreover, even the 5-HT pathway of TRP metabolism may influence the KYN route: a notable example could be the reported effect of melatonin on TRP metabolism by inducing IDO and increasing its activity. Noticeably melatonin, which seems also able to exert an anti-inflammatory action and to stabilize the gut barrier, is well-known for its role in regulating circadian rhythmicity and sleep, biological functions that appear often compromised in ASD (Li et al. 2017; Savino et al. 2020). The activation of the KYN pathway will lead not only to an increased synthesis of its metabolites, but also to TRP depletion and to the reduction of TRP metabolism through the methoxyindole route (Bilgiç et al. 2020).

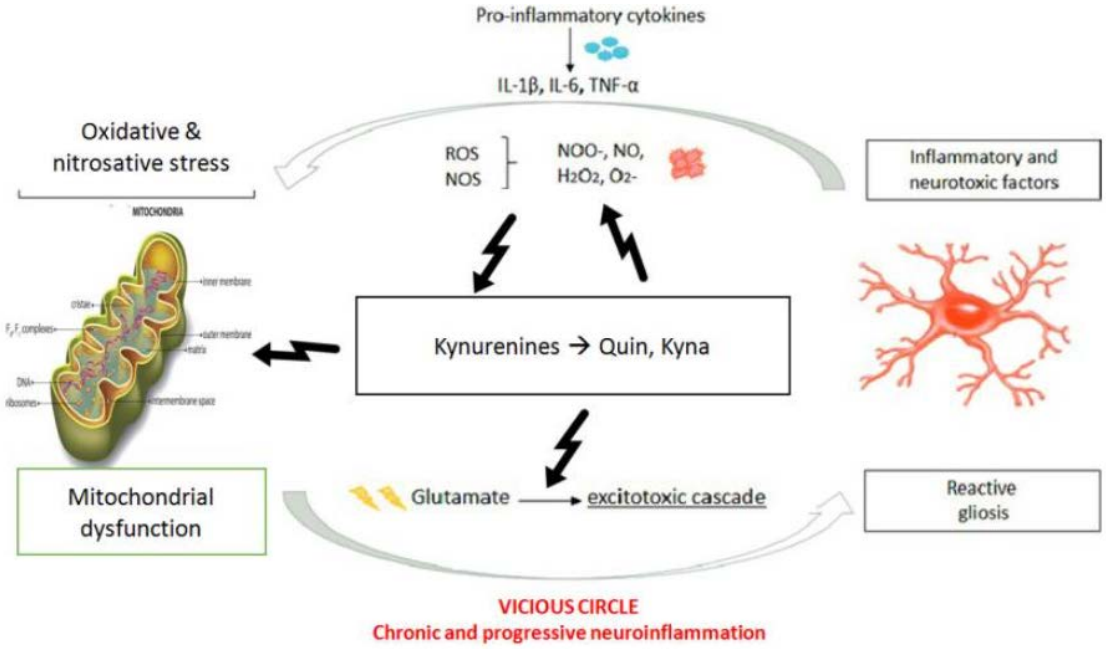

Figure I3.The role of inflammation in shifting TRP metabolism through KYN pathway, activating IDO and KMO enzymes. The excitotoxic effects of QUIN through the activation of NMDA receptors, the glutamate increase and the increase of oxidative stress would lead to mitochondrial dysfunction, further promoting, in a vicious circle, neuroinflammation (from Savino et al. 2020).

Blood KYN levels were reported to be correlated to the CSF ones, while most of the brain KYN seems to come from the periphery. As a consequence, the peripheral levels of the metabolites of the KYN pathway may reflect their asset in the CNS (Bilgiç et al. 2020). In this framework, D'Eufemia et al. (1995) stressed the importance of evaluating the ratio between TRP and the sum of the other LNAAs (TRP/LNAAs), which use the same transporters of TRP for passing through the BBB, because it would be the principal factor that influences the TRP uptake in the brain and therefore its availability for 5-HT synthesis in the CNS. They also reported, in a sample of 46 ASD children, a lower TRP/LNAAs ratio when compared with 40 healthy children.

TRP is also a target of nitrosative and oxidative stress produced by the activation of the immune system. In particular, TRP can be subjected, as other aromatic amino acid, to nitrosylation, becoming nitroso-TRP (Ormstad et al. 2018).

Another important aspect to consider is that TRP levels can also be modulated by the gut microbiota. Amounts of diet-deriving TRP can reach the large intestine and be

degraded in tryptamine through decarboxylation processes or be transformed to indole and its derivatives by microbes (Gao et al. 2019) (see **figure I4**). It has been hypothesized that TRP may play a central role in the gut brain axis (GBA) and in the crosstalk between microbiota and the CNS: microbiota (and its variations) may affect CNS modulating TRP concentration and that of its metabolites of both methoxyindole and KYN pathways. Microbiota seems to also modulate peripheral 5-HT concentrations, promoting 5-HT synthesis (or even directly synthesizing it) from TRP. Moreover, some metabolites of the gut microbiota seem to influence IDO activity, while microbiota may also modulate the KYN pathway through its crosstalk with the immune system, which, in turn, may activate the KYN route. In mouse models lacking gut microbiota, which show an immune system deficiency, authors reported reduced TRP metabolism through the KYN pathway (Clarke et al. 2013; Gao et al. 2019). In this framework, it should be noted that both microbiota and immune system alterations were reported among ASD subjects (Carpita et al. 2020a).

In summary, TRP metabolism is a metabolic route that produces small molecules, methoxyindoles and KYN derivatives, interacting as signals of redox or pro-apoptotic/anti-apoptotic cellular/neuronal responses, the impairment and imbalance of which can be related to several conditions of brain/mental dysfunction, including ASD (Savino et al. 2020).

TRYCAT pathway alterations were found in several psychiatric and neurological disorders other than ASD (Savino et al. 2020). Specifically, an activated TRYCAT pathway was reported in Multiple sclerosis, Alzheimer's and Parkinson's disease, psychosis and Schizophrenia (Ormstad et al. 2018; Bilgiç et al. 2020). This metabolic path was also linked to anxiety, Somatic symptom disorder and depression (Songtachalert et al. 2018; Bilgiç et al. 2020). Recent meta-analyses on KYN metabolites reported lower peripheral levels of TRP, KYN and KYNA in both Bipolar disorder and Major depressive disorder, while lower peripheral levels of TRP and KYN were reported in Schizophrenia (Marx et al. 2020; Hebbrecht et al. 2021). According to another meta-analysis (Morrens et al. 2020), KYNA and QUIN in Schizophrenia would be decreased in patients with acute symptoms, while KYN and KYNA would be decreased in older patients. Moreover, Marx et al. (2020) also highlighted different patterns between mood disorders and Schizophrenia, with a higher tendency towards the production of QUIN instead of KYNA only in mood disorders. According to their

findings, a lower KYNA/QUIN ratio would be detectable only in mood disorders, while lower KYNA/KYN and increased KYN/TRP would be found in both Schizophrenia and Major depressive disorder (Marx et al. 2020).

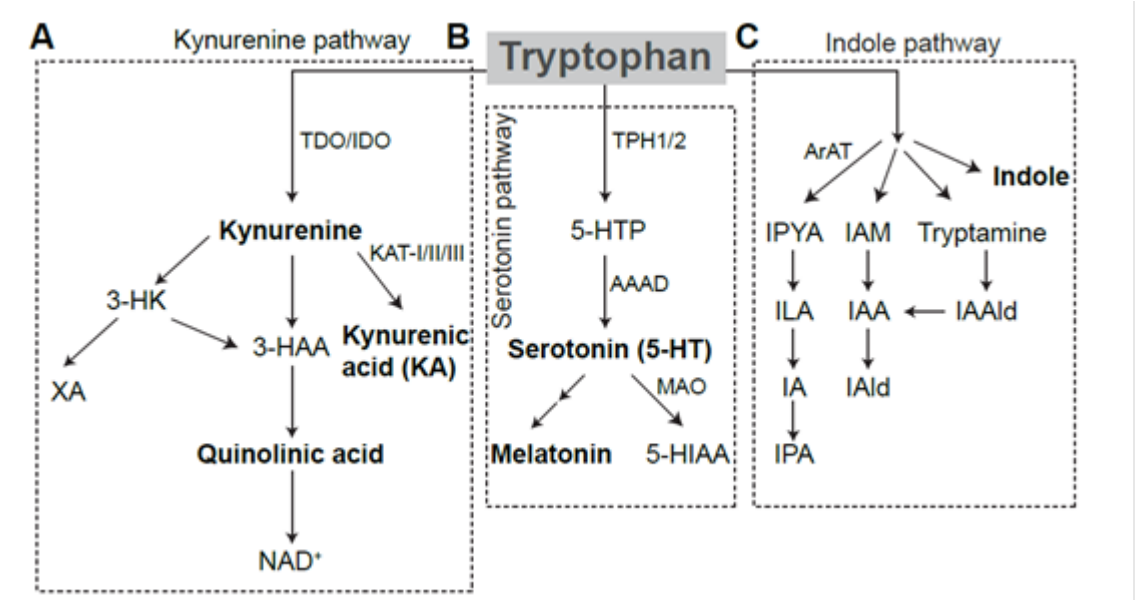

Figure I4. TRP metabolic pathways. In the host TRP metabolism may occur through the KYNA or the 5-HT pathway. In gut microbes, which express different enzymes, TRP is metabolized in indole and derivatives. 3-HAA: 3-hydroxyanthranilic acid; 5-HIAA: 5-hydroxyindoleacetic acid; 3-HK: 3-hydroxykynurenine; 5-HTP: 5-hydroxytryptophan; AAAD: aromatic amino acid decarboxylase; ArAT: aromatic amino acid aminotransferase; IA: anholocyclic acid; IAA: indole-3-acetic acid; IAAld: indole-3-acetaldehyde; IAld: indole-3-aldehyde; IAM: indole-3-acetamide; IDO: indoleamine 2,3-dioxygenase; ILA: indole-3-lactic acid; IPA: indole-3-propionic acid; IPYA: indole-3-pyruvic acid; KAT: kynurenine aminotransferase; MAO: monoamine oxidase; NAD: Nicotinamide adenine dinucleotide; TDO: tryptophan 2,3-dioxygenase; TPH: Tryptophan hydroxylase; XA: xanthurenic acid. (from Roth et al. 2021).

It should be noted that some studies reported increased levels of KYNA in the CSF of patients with Schizophrenia and Bipolar disorder (in particular, for this latter, among subjects with psychotic features), hypothesizing the presence of higher central levels of KYNA, but decreased peripheral ones, in these populations (Kindler et al. 2020;

Hebbrecht et al. 2021). Among schizophrenic patients, it was theorized that increased central levels of KYNA might be responsible of cognitive deficits through the inhibition of NMDA and cholinergic receptors, with a reduced basal glutamate synapsis activity as a result (Banerjee et al. 2012; Bilgiç et al. 2020). This hypothesis would be in contrast with the one suggesting that reduced KYNA levels would underlie a reduced neuroprotective effect, leading to brain damage due to an increased excitotoxicity (van den Ameele et al. 2020; Kindler et al. 2020; Hebbrecht et al. 2021). Increased QUIN and KYN levels have also been associated with suicidal ideation/behaviors (Erhardt et al. 2013; Sublette et al. 2011; Lim et al. 2016; Bryn et al. 2017).

More controversial results were reported for 3-HK in Schizophrenia and mood disorders, with no difference reported in meta-analytic studies (Hebbrecht et al. 2021; Morrens et al. 2020). 3-HAA is another metabolite hypothesized to play a role in several disorders, from stroke and osteoporosis to depression, although it is not clear whether reduced 3-HAA levels may increase inflammation processes or exert a compensatory function reducing toxicity in brain cells (Miller et al. 2008; Darlington et al. 2010; Aarsland et al. 2015; Teshigawara et al. 2019; Bilgiç et al. 2020). Only few studies investigated KYN pathway in patients with Attention deficit hyperactivity disorder (ADHD): one study in adults reported lower TRP, KYNA and 3-HAA levels, while in children were reported instead increased levels of TRP by one study, together with reduced levels of KYNA. 3-HK was alternatively found reduced or not different in patients vs. controls (Evangelisti et al. 2017).

In the field of ASD, increasing interest is paid on TRP metabolism: some authors have even stated that altered TRP metabolism might be considered “the unifying biochemical basis for ASD” (Schwartz et al. 2014). Despite that, studies focusing on TRP and, specifically, KYN pathway in ASD are still limited. Considering TRP itself, most of the studies were conducted on ASD children, often reporting reduced plasma TRP levels in ASD children than in controls (Tirouvanziam et al. 2011; Tu et al. 2012; Naushad et al. 2013; Zheng et al. 2017). On the other hand, Hoshino et al. (1984) reported increased plasma TRP and 5-HT levels in ASD children, although without finding a correlation between the two markers. These authors also stressed how TRP availability in the brain might be linked to the free plasma levels of this amino acid. Among older studies, Minderaa et al. (1989), who evaluated TRP levels in an older sample of subjects, did not find differences between ASD patients (n = 40, mean age = 19.4±4.9 years) and controls

(n = 20, mean age = 22.0±7.5 years). Croonenberghs et al. (2000) focused on post-puberal ASD patients (n = 13, age range = 12-18 years), with an Intelligence Quotient (QI) > 55, reporting lower plasma TRP concentrations in the ASD group than in controls, but no difference in 5-HT levels, as measured in PPP, PRP or serum. However, [^{3}H]-paroxetine binding K_D values were found higher in the ASD group, suggesting a reduced affinity of platelet [^{3}H]-paroxetine binding sites, eventually linked to conformational states of the SERT protein. Boccuto et al. (2013) mixed subjects of different age (age range = 2.5-34.25 years) and evaluated the metabolic profile of lymphoblastoid cell lines among patients with ASD (n = 87), other neurodevelopmental disorders (n = 40) or Schizophrenia (n = 10), and in controls (n = 78). Interestingly, when using TRP as the unique energy source, they highlighted a reduced NADH generation, which is under the control of the 5-HT branch of TRP metabolism, only in the ASD group. This suggests an altered TRP metabolism in these patients, in particular their reduced ability to use the amino acid as an energy source.

In urine samples, more recently some authors found increased TRP concentrations among ASD patients (Noto et al. 2014), while others reported decreased TRP levels (Kałużna-Czaplińska et al. 2014). Gevi et al. (2016) found that TRP, together with purines, was one of the urinary metabolites which displayed greater differences in a sample of ASD children when compared with the control group. Specifically, ASD children appeared to preferentially metabolize TRP into xanthurenic acid and QUIN, with a reduction of the KYNA and melatonin routes. They also highlighted how, in their subjects' sample, the gut microbiota was exerting a significant influence on TRP metabolism by promoting an increase of indolyl 3-acetic acid and indolyl lactate. On the basis of these results, they hypothesized that ASD subjects would have a cell danger response featuring an increase of the neurotoxic intermediate QUIN and a reduction of melatonin, together with a condition of gut dysbiosis. The authors also highlighted how these alterations might be in line with the multiple comorbidities often associated with ASD, such as gastrointestinal disorders and sleep alterations. Kałużna-Czaplińska et al. (2017) found, in a wide sample of ASD subjects, significant differences in urine TRP concentrations depending on ASD severity: in particular, subjects with Infantile autism showed higher levels than subjects with Asperger syndrome. They also found that patients taking supplementation of B vitamins and magnesium showed lower urinary

levels of TRP when compared with the others, without significant difference depending on the BMI (Body Mass Index, kg/m^2).

Among ASD patients, a reduced expression of the *TPH2* gene was also underscored, (Boccuto et al. 2013; Kałużna-Czaplińska et al. 2017), together with genetic variations of the NMDA receptors gene. Polymorphisms of the gene encoding some subunits of the LAAs transporters (LAT1 and LAT2), involved in TRP transport across the BBB, as well as of the *TPH* gene were reported in ASD subjects (Savino et al. 2020).

In mouse models, increased dietary TRP supplement has been shown to promote social behaviors, worsened instead by a reduced TRP intake (Zhang et al. 2015; Zheng et al. 2017). Moreover, higher IDO expression was associated with immune disorders and inflammation in animal models, whereas, on the contrary, acute stress and immune system activation may increase TRP (Wirthgen et al. 2016, Kałużna-Czaplińska et al. 2017; Savino et al. 2020). On the other hand, an increased supplementation of dietary TRP seems to reduce aggressive behaviors and cortisol levels after stressful stimuli (Savino et al. 2020).

Human studies have reported a link between deficits in specific cognitive abilities, such as episodic memory consolidation for verbal information, and acute TRP depletion (Mendelsohn et al. 2009; Savino et al. 2020). Among patients with ASD (both adults and children) dietary TRP deprivation was reported to worsen ASD symptoms (McDougle et al. 1996; Lim et al. 2016; Savino et al. 2020), while acute depletion of TRP seems to inhibit 5-HT synthesis (Nishizawa et al. 1997; Savino et al. 2020).

To date, only few studies have been conducted on TRP catabolites and the KYN pathway in ASD subjects, possibly due to controversies and inconsistencies found in first studies in this field. Zimmerman et al. (2005) did not find differences in CSF QUIN contents between ASD children and controls, although control subjects in this study were affected by other neurological disorders. Sweeten et al. (2006) did not report significant differences in plasma KYN levels between ASD children and controls, hypothesizing that the KYN pathway should not be considered involved in ASD. Conversely, more recently, Lim et al. (2016) investigated 15 Omani families where ASD children were present, by using their age-matched healthy siblings as a control group. This study reported an increased KYN/TRP ratio, increased QUIN concentrations, as well as reduced PA in ASD patients. Noticeably, PA is another KYN shunt molecule exerting neuroprotective functions (Bryn et al. 2017). The authors

emphasized that the lack of TRP quantification in the study conducted by Sweeten et al. (2006) may have led to misleading results due to the high dynamicity of TRP and KYN concentrations and the importance of evaluating the ratio of these two components, instead of only their single concentrations, as a valuable index of KYN pathway activation (Lim et al. 2016). Lim et al. (2016) also hypothesized that an increased KYN/TRP ratio may reflect IDO activity, in particular the induction of the IDO-1 enzyme, which may be triggered by several cytokines, implying a potential involvement of neuroimmune mechanisms in ASD pathogenesis. Moreover, high QUIN concentrations may suggest the presence of higher oxidative stress: considering that macrophages are believed to be the main cell lineage responsible of QUIN production, it was hypothesized that the triggered status of KYN metabolism might be part of innate immunity in response to the elevation of pro-inflammatory mediators. According to these authors, several other reports have highlighted increased levels of oxidative stress markers and various cytokines among ASD children, accompanied by reduced levels of antioxidants, such as glutathione (Lim et al.2016). In light of a previous research which reported reduced NAD^+ levels in ASD children, the finding of increased QUIN in this population may suggest an impairment of QUIN catabolism (Essa et al. 2013; Lim et al. 2016). Another study, conducted by Bryn et al. (2017), investigated serum levels of TRP, KYN, KYNA, QUIN and 3-HK in a sample of 65 children with ASD and 30 healthy children, reporting significantly lower KYNA levels and higher KYN/KYNA in the ASD group. They found similar results when they compared the Childhood autism sub-group with the control group. They also found significantly lower TRP values in ASD subjects with a previous diagnosis of Childhood autism than in those previously diagnosed with Asperger syndrome. The authors suggested that an increased KYN/KYNA ratio may imply the presence of an increased neurotoxic potential in the patients' group (linked to the KYNA activity as an agonist of the NMDA receptor), while a reduced KAT activity may be responsible for the reduced levels of KYNA, as previously reported in neurodegenerative disorders such as Alzheimer's and Parkinson's disease (Bryn et al. 2017). KYN/KYNA may provide information on KAT activity and the neurotoxic potential of this path flux, the QUIN/KYNA ratio may rather reflect the excitotoxicity of NMDA receptor activation (Bryn et al. 2017). Another study further evaluated this topic by focusing on serum levels of TRP, KYN, KYNA, Brain-derived neurotrophic factor (BDNF) and PRO-BDNF, together with levels of 5-HT and 5-HTP

among ASD children (n = 65, mean age = 11.2±2.02 years) and controls (n = 30, mean age = 10.9±2.1 years) (Ormstad et al. 2018). Specific attention was paid in comparing ASD subjects of different subtypes. In particular, they found lower TRP and KYNA levels, together with increased BDNF amounts, among subjects with Childhood autism and Intellectual disability disorder (IDD), and higher levels of TRP and lower 5-HT synthesis in Asperger syndrome. In the Childhood autism group, these findings seem to be associated with the concomitant presence of IDD. Subjects showing abnormalities by means of brain imaging technologies, such as Magnetic resonance imaging (MRI), had higher TRP and lower KYNA levels, this latter data being also associated with the presence of gastrointestinal symptoms. An altered electroencephalography (EEG) profile was found related to increased BDNF/PRO-BDNF ratio. More recently, other authors specifically investigated the KYN pathway in ASD children aged between 18 and 60 months, on the basis that early childhood was known to be a critical period for ASD onset, and that, on the other hand, KYN metabolism was considered to be age-dependent (Sorgdrager et al. 2019; Bilgiç et al. 2020). In contrast to previous results, this study also evidenced increased serum 3-HK and KYNA levels with decreased 3-HAA in ASD patients vs. controls of the same age. No difference was found for TRP and KYN and the ratios of the examined metabolites. The other study which evaluated 3-HK levels in ASD was that of Bryn et al. (2017), which did not find significant differences between patients and controls (Bryn et al. 2017). However, the authors hypothesized that increased 3-HK levels may be involved in ASD pathogenesis, considering that, in neuronal cultures, this finding was associated with oxidative stress, apoptosis and mitochondrial dysfunction. Moreover, they also underlined that, as previously hypothesized for Schizophrenia, increased KYNA levels in ASD may imply a glutamatergic and cholinergic dysfunction, which eventually would lead to cognitive deficits (Bilgiç et al. 2020).

Finally, it should be pointed out that research on TRP metabolism in ASD, particularly on the KYN pathway, is still limited, showing a specific lack of studies addressing adult samples and/or the issue of BAP, including the scarce investigation on altered KYN pathway and its potential link with sub-threshold autistic traits.

1.4 Autism spectrum and immune system alterations

Since 1977 a high heritability and genetic influence have been highlighted in ASD by studies on twins (Folstein and Rutter 1977; Ronald and Hoekstra 2011), corroborated by the presence of BAP in close relatives of ASD patients. Starting from the hypothesis of a possible interaction between genes and environment in shaping ASD pathogenesis, an increasing interest has been paid to environmental factors which could eventually play a role through epigenetic modifications, mostly occurring during intrauterine life (Carpita et al. 2020a). Several authors focused on the potential involvement of other endogenous systems which, acting at the interplay between environmental and genetic factors in response to stimuli or stressors, may influence ASD neurodevelopmental trajectory (Siniscalco et al. 2013; 2018; Carpita et al. 2020a).

In this framework, many studies focused on possible immune system alterations in ASD. The interest in evaluating immune response and inflammatory status in this population rose in the '70, starting from the several reports of comorbid autoimmune disorders in ASD children, who would be more often affected by somatic diseases such as diabetes, inflammatory bowel diseases, allergies and asthma (Zimmerman et al. 2007; Ruggeri et al. 2014; Sakamoto et al. 2015; Li and Zhou 2016; Kern et al. 2016; Onore et al. 2012; Carpita et al. 2020a). ASD and immune system alterations may share common genetic bases, considering that a family history of autoimmune disorders was also reported in ASD children (Onore et al. 2012; Carpita et al. 2020a). On the other hand, among pregnant mothers of children with ASD, some authors showed an increased history of infections or fever in the first trimester (Doenyas 2018), as well as the presence of brain-reactive autoantibodies, leading to hypothesize a possible role of maternal autoantibodies in ASD pathogenesis (Bjorklund et al. 2016; Siniscalco et al. 2018). Moreover, even maternal gestational diabetes, which can lead to higher levels of oxidative stress, inflammation and immune activation, was identified as a potential risk factor for ASD in the offspring (Chen and Scholl 2005; Biri et al. 2006; Gardener et al. 2009; Krakowiak et al. 2012; Li et al. 2016; Carpita et al. 2018). Similar results were reported for pre-eclampsia and obesity (Carpita et al. 2018). In animal models, a high-fat diet, also linked to oxidative stress and pro-inflammatory states, has been associated with altered offspring neurodevelopment (Bilbo and Tsang. 2010; Carpita et al. 2018). This line of studies recently gained more attention, after the pivotal finding that, in

patients with Schizophrenia, the relationship between genetic vulnerability and the actual development of the disorder may be modulated by the intrauterine environment, specifically through the up-regulation of specific genes expressed in the placenta in response to stress during gestation (Ursini et al. 2018; Carpita et al. 2020a).

Moreover, an enhanced inflammatory activity, that may be linked to immune system dysfunctions, was reported among ASD patients (Siniscalco et al. 2018; Carpita et al. 2020a). Several studies focused on the comorbidity between ASD and autoimmune disorders from an epidemiological point of view, while others evaluated biochemical correlates of inflammatory activity and/or immune system deregulation in these patients (Siniscalco et al. 2013; Carpita et al. 2020a). One of the first parameters investigated in this field were immune cells. For instance, natural killer (NK) cells seem to show a reduced responsivity in ASD patients, while their number would be increased (Vojdani et al. 2008; López-Cacho et al. 2016; Bjorklund et al. 2016; Siniscalco et al. 2018). Other reports showed an increased number of monocytes and B cells in ASD children, as well as reduced levels of CD4+T and of CD4+/CD8+T cells ratio, although not all the studies replicated these results (Bjorklund et al. 2016; López-Cacho et al. 2016; Carpita et al. 2020a). In adult subjects with ASD a study reported instead a reduced number of NK cells and a higher one of B and CD8+T cells (López-Cacho et al. 2016).

Considering immunoglobulins (Ig), results from literature are contrasting: authors reported alternatively reduced or increased levels of IgG and IgM (Gupta et al. 1996; Croonemberghs et al. 2002a; Heuer et al. 2008) in these patients, with some studies also highlighting an association between Ig levels and symptoms severity (Piras et al. 2014). The presence of different kinds of autoantibodies able to react against CNS was also reported in ASD children (Cabanlit et al. 2007; Wils et al. 2007; Onore et al. 2012; Mostafa et al. 2016; Carpita et al. 2020a).

In the last years, most of the studies in this field focused on cytokines, and specifically on interleukins. Cytokines have been thought as promising biomarkers of ASD, since these proteins are a source of information on the state of the immune system relatively simply to measure. Moreover, they can also directly affect the CNS, being potentially implied in the pathophysiology of ASD (Onore et al. 2012; Carpita et al. 2020a). Pro-inflammatory cytokines such as IL-1α, IL-1β, IL-6 and TNF-α, are recognized as sickness signals, while IL-1β, TNF-α, IL-6 may also pass through the BBB and directly exert their effect on hypothalamus (Masi et al. 2017). A model of how cytokines may

affect CNS was identified in “sickness behavior”, which features social withdrawal, appetite reduction, fatigue, irritability, and is also considered an adaptive response facilitating fever and other biological mechanisms for fighting infections. Thus, according to this model, the immune system would be able to directly affect the CNS producing behavioral changes as a part of the response against infections/immune challenges (Masi et al. 2017) (see also **figure I5**).

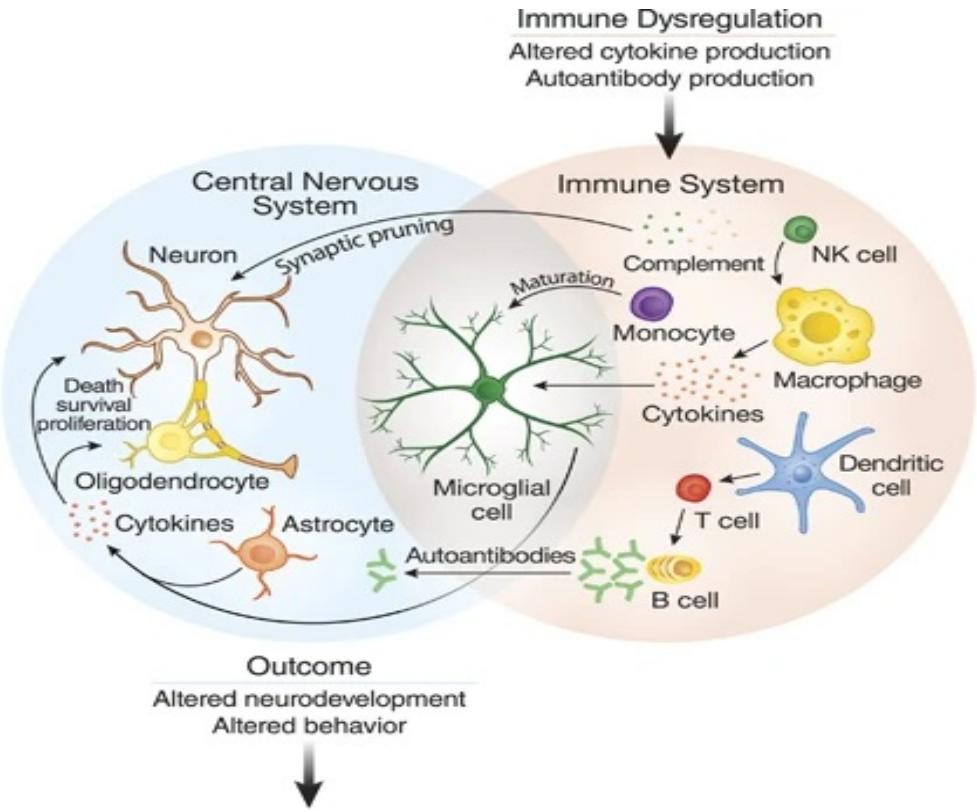

Figure I5. The complex network of interactions through which the immune system may affect neurodevelopment and behavior. Microglia cells and complement protein can affect synaptic scaling and pruning, T- and N-cells can show altered activity and reactivity, some cytokines can regulate the neural cell balance through neurogenesis or neural death. Brain-reactive antibodies may instead affect the neuronal development or function (from Meltzer and Van de Water 2017).

Moreover, peripherally activated macrophages may also enter the CNS parenchyma, contributing to further release cytokines, or, alternatively, peripheral signals of cytokines may be transmitted by afferent nerves (Bauman 2010; Mannion and Leader 2013; Masi et al. 2017). Noticeably, sickness behavior and depression share several symptoms: on these bases increasing research stressed the hypothesis of an involvement

of cytokines also in psychiatric disorders' pathophysiology (Masi et al. 2017), which seems to be confirmed by the presence of altered cytokine patterns in mood disorders and Schizophrenia (Dowlati et al. 2010; Miller et al. 2011; Modabbernia et al. 2013; Masi et al. 2017).

Cytokines are proteins with signaling functions, mainly showing a size lower than 80kDa. They coordinate the innate and acquired immune response, the immune system reactivity to infection and other immune challenges, being responsible of the communication between the different cells of the immune system and associated processes, including the induction of the synthesis of other cytokines. They are involved in inflammation and repair, but also in hematopoiesis and cell proliferation. While the same cytokine may exert multiple effects on different targets (pleiotropism), different cytokines may have the same function, a mechanism called redundancy (Chung 2009; Masi et al. 2017). Cytokines are produced by different cell types, although some of them may have a principal source from a specific cell line. Granulocyte (G) macrophage (M) colony-stimulating factor (GM-CSF) may be produced by macrophages, fibroblasts and endothelial cells. T helper (Th) lymphocytes (or CD4+T) type 1 cells are the main responsible for the production of interferon-γ (IFN-γ), IL-2, tumor necrosis factor-β (TNF-β), TNF-α. Th2 cells instead produce IL-4, IL-5, IL-6, IL-9, IL-10, IL-13 (Masi et al. 2017). Lymphocyte Th cells mediate the adaptive immunity, and they are differentiated depending on their function. Th1 cells mainly produce INF-γ, IL-2 and TNF-β, activate macrophages and cell-mediate immunity, as well as the phagocyte-dependent protective response. They are involved in the response to infection by intracellular bacteria and viruses. Th2 cells are involved in the response to infestation by parasites and, producing IL-4, IL-5, IL-10 and IL-13, activate the eosinophils and the antibody production, but also inhibit macrophages, leading to a phagocyte independent protective response (Romagnani 1999). The balance between Th1 and Th2 is of the utmost importance for immune regulation, while the over-activity of one Th type may suppress the other kind of immune response (Masi et al. 2017). From an immunopathological point of view, Th1 cells seem to be involved in autoimmune disorders that are organ-specific, while Th2 cells, through allergen-specific responses, are involved in atopic disorders (Romagnani 1999).

Among cytokines, a specific sub-group can be identified in chemokines, which are able to recruit, by chemical signals, specific sets of leukocytes. Cytokines can be classified

in several different ways. They have been classified in six groups depending on their structure and/or the structure of their receptors. The wider group is the Hematoprotein family, whose members have a 4-helix bundle structure, although they differ in functions and effects, from proliferation to secretion of antibodies. This family includes several interleukins, such as IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-12p40, IL-12p70, IL-13, IL-15, IL-23, GM-CSF, G-CSF. Other groups are the IL-1 family (linked to mainly pro-inflammatory response to infections); TNF family, linked to apoptotic mechanisms; interferon family (IL-10, IFN-γ), which is linked to antiviral response, and exerts its action supporting the generation of Th1 cells and the activation of macrophages; IL-17 family, with mainly pro-inflammatory effects, and chemokine family - including IL-8, eotaxin, monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1α and -1β (MIP-1α, MIP-1β) -, which mediates the secondary pro-inflammatory response, promotes chemo-attraction and the recruitment of specific groups of lymphocytes (Masi et al. 2017). Depending on the distance of the range of action, cytokines may act through: 1) an endocrine mechanism, if they must cross the bloodstream in order to reach the receiving target; 2) a paracrine mechanism, if their function is exerted near the secreting cell; 3) an autocrine one, if they affect the same cells from which they have been secreted (Masi et al. 2017). Moreover, depending on the kind of immune response in which they are involved, they could be divided in anti-inflammatory, pro-inflammatory, and adaptive immunity-related cytokines (Turner et al. 2014; Masi et al. 2017). While cytokines such as IL-1, IL-12, IL-18, GM-CSF, TNF and IFN-γ are usually considered pro-inflammatory, IL-4, IL-10, IL-13, interferon-α (IFN-α), transforming growth factor-β (TGF-β) were considered anti-inflammatory ones. However, it should be noted that several authors pointed out how the same cytokine may exert pro-inflammatory and anti-inflammatory actions depending on the target cells, the activating signal, the nature of the produced cytokines, the timing and the cytokine amount (Cavaillon 2001; Masi et al. 2017). IL-6 in particular, although often considered a pro-inflammatory cytokine, may also exert an anti-inflammatory action. Moreover, IL-6 was reported to be involved not only in the inflammatory response but also in metabolic, neural and regenerative processes. It is a member of a wider cytokine family, the IL-6-like cytokines, which gained in literature the name of neuropoietic cytokines due to their role in mediating neuronal survival and differentiation (Gadient and Otten 1997). Other members include IL-11, leukemia inhibitory factor (LIF), ciliary

neurotrophic factor (CNTF), oncostatin M, cardiotrophin-1 (CT-1) and growth promoting activity (GPA). They all show a tertiary structure of four antiparallel α-helices, leading to very similar 3-D forms, and their molecular weight in the non-glycosylated form is around 20-24kDa. All family members are pluripotent and involved in several processes, often inducing similar biological responses (Gadient and Otten 1997). Indeed, functional redundancy is a main feature of cytokines. It should be noted that IL-6, in particular, seems to be involved in shaping synaptic connections (Siniscalco et al. 2018), whereas increased CNS levels of IL-6 were reported to affect neural cell adhesion and migration in ASD, eventually impairing the synapse formation processes (Zhao et al. 2021).

IL-6 may affect its target cells by classical signal transduction pathways or, alternatively, by trans-signaling processes. In the first, IL-6 activates target cells through the binding to its receptor, which is membrane-bound and coupled to the signaling receptor protein gp130 (Scheller et al. 2011). However, only a limited number of cells express IL-6 receptors, although they may still express gp130. Through the trans-signaling mechanism, IL-6 forms, instead, a complex with a soluble form of its receptor, and subsequently this complex is able to stimulate also cells which express only gp130, greatly amplifying the potential targets of this cytokine. It was also reported that a releasable form of gp130 (sgp130) may exert an inhibitory effect on trans-signaling (Erta et al. 2012). It has been hypothesized on the basis of some evidence that pro-inflammatory actions of IL-6 would be mediated by trans-signaling, while anti-inflammatory/regenerative actions would be mediated by the classic signaling (Scheller et al. 2011) (see also **figure I6**).

During inflammation, IL-6 is produced by cells of the innate immune system when a damage-associated or pathogen-associated molecular pattern (DAMP or PAMP) is detected. Moreover, it can be released by endothelial cells during acute inflammation processes in order to attract neutrophils, and, subsequently, to promote neutrophil apoptosis, contributing to the resolution of acute neutrophil infiltration and, at the same time, switching from neutrophil to monocyte recruitment in the site of inflammation. IL-6 is also able to promote monocyte differentiation, enhancing the expression of M-CSF receptor, and to up-regulate cell adhesion molecules on endothelial cells.

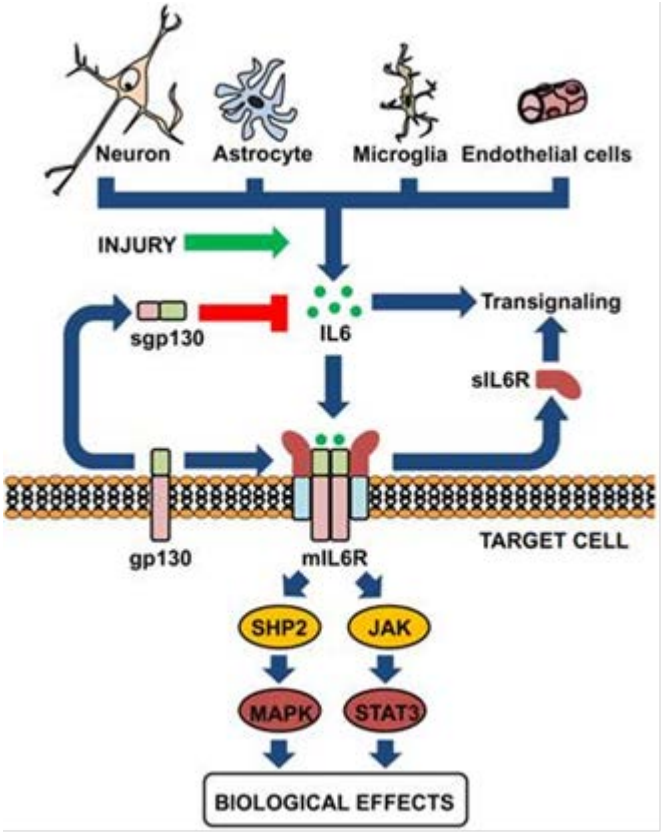

Figure I6. IL-6 production and receptor (sIL-6R) binding through classic signaling and trans-signaling (from Erta et al. 2012).

Moreover, it is involved in the recruitment and differentiation of B and T cells. IL-6 protects T cells from apoptosis and promotes the production of antibodies by the B cells, and it seems to skew the differentiation of T cells towards Th2 and Th17, as well as to inhibit the differentiation into regulatory T cells. While Th17 cells are involved in the induction of autoimmunity, regulatory T cells seem to be able to protect from tissue injury and inhibit autoimmunity processes (Scheller et al. 2011). Studies in rodent models highlighted a role of IL-6 in pain modulation. IL-6 seems to sensitize heat nociceptors in skin and to modulate, in sensory neurons, the ionic current activated by heat, inducing heat hypersensitivity. These studies also support the use of neutralizing agents of IL-6 receptors in patients with diseases associated with pathological pain, such as rheumatoid arthritis (Scheller et al. 2011). Noticeably, IL-6 seems to be produced also by adipocytes in obesity and by muscle cells during exercise (Pedersen et al. 2003; Ghoreschi et al. 2010; Scheller et al. 2011). The complex formed by IL-6 and its soluble

receptor is able to trigger the formation of osteoclasts, possibly by inducing RANK ligand expression, which is responsible for stimulating osteoclastogenesis (Hashizume et al. 2008; Scheller et al. 2011). A role of IL-6 in metabolism was hypothesized by many authors: the lack of IL-6 is associated with insulin resistance and liver inflammation in mice, while in humans the intake of IL-6 receptor-neutralizing agents, such as tocilizumab, can be associated with dyslipidemia and weight gain (Scheller et al. 2011). In the brain, IL-6 is responsible, together with IL-1β, of promoting the so-called "sickness behavior", displaying pyrogenic and corticotropic activities, and also potentiating the depressive-like symptoms. Although it was reported that, among cytokines, IL-1β seems to be the main responsible for the typical behavioral changes linked to the "sickness state", it should be noted that depression has been associated with an increase of pro-inflammatory cytokines and in particular IL-6, eventually implying a role of IL-6 in potentiating the behavioral effects of IL-1β (Dantzer 2009; Dowlati et al. 2010). It was also found that sympathetic and sensory ganglia, as well as adrenal chromaffin cells express IL-6 and IL-6 receptor transcripts in adult rats, where IL-6 seems to be able to promote the survival of sensory neurons (although the same activity was not reported towards adrenal chromaffin cells), with a potential neurotrophic role in the peripheral nervous system (Gadient and Otten 1997). Moreover, in several brain areas, including the neocortex, cerebellum and hippocampus, neurons and astrocytes have been found to express IL-6/IL-6 receptor transcripts. In both periphery and CNS, the mRNA of IL-6 and IL-6 receptor seems to be developmentally regulated, accumulating during growth, at least in some regions. Besides the above-reported effects involved in sickness behavior, CNS IL-6 has been related to neuronal survival and differentiation as well as to astrocyte proliferation, in order to prevent neuronal damage by also modulating the intracellular calcium response to NMDA, and to regulate neurotransmitter biosynthesis (Gadient and Odent 1997). On the other hand, animal models overexpressing IL-6 in the CNS reported increased glial proliferation, neovascularization, BBB disruption, induction of acute phase proteins and global neurodegeneration, in particular in the cortex and in the cerebellum (Campbell et al. 1993; Gadient and Odent 1997). In addition, besides psychiatric disorders, also several neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases, or inflammatory/autoimmune disorders such as multiple sclerosis, are linked to an increase of IL-6. However, it is not clear if the increased IL-6 levels should be considered to

have a causative effect, a compensatory effect or to be a parallel process linked to the ongoing inflammation (Gadient and Otten 1997). In animal models of autoimmune encephalitis, IL-6 is greatly up-regulated and accumulates in the CNS, possible due to microglia activation and invasion of monocytes. The increase of IL-6 in the CSF may be underlain by an increased IL-6 synthesis by local glial and neuronal cells, or eventually by an IL-6-producing mononuclear cell infiltration through the CNS parenchyma, such as monocytes or T cells. Moreover, it is also possible that IL-6 produced in the periphery may reach the CSF passing through the BBB (Gadient and Otten 1997).

Overall, IL-6 seems to play an important role in mediating the communication between the immune system and the CNS. Due to the reported relevant effects of IL-6, this cytokine is one of the most investigated in psychiatry, in particular in mood disorders (Goldsmith et al. 2016).

Increased IL-6 levels were also reported to be associated with altered sleep-wake cycles and poor sleep quality (Masi et al. 2015; Saghazadeh et al. 2019a; Zhao et al. 2021). Noticeably, cognitive deficits were reported in *IL-6/IL-4 knockout* mouse models (Baier et al. 2009; Derecki et al. 2010; Onore et al. 2012; Carpita et al. 2020a). Other studies on animal models stressed an association between TNF-α, IL-1β and social withdrawal, which was also hypothesized to be, from an evolutionary point of view, a possible adaptive behavior in response to sickness state (Harden et al. 2008; Onore et al. 2012; Carpita et al. 2020a). In the field of ASD, one of the most frequent findings was the presence of increased levels of pro-inflammatory cytokines and reduced levels of the anti-inflammatory ones, in both the CSF and in the peripheral tissues of patients (Bjorklund et al. 2016; Siniscalco et al. 2018; Carpita et al. 2020a). Moreover, an over-production of pro-inflammatory cytokines after a challenge test by endotoxin (or lipopolysaccharide, LPS: the outer cell wall membrane of Gram-negative bacteria) was reported in ASD patients (Siniscalco et al. 2018).

The great majority of cytokine studies were conducted in ASD children, but results remain controversial, probably due to a high heterogeneity in the methods of the available researches: different kinds of instruments were used for ASD assessment, the cytokines investigated often vary, and also the biological specimens in which they are measured (Masi et al. 2015; Bjorklund et al. 2016; Masi et al. 2017; Siniscalco et al. 2018). Considering the works in this field, among the various cytokines, in particular IL-1β, IL-5, IL-6, IL-8, IL-12p40, IL-12p70, IL-13, IL-17 and TNF-α levels were

reported to be higher in ASD children (Al-Ayadhi 2005; Ashwood et al. 2011a; Brocker et al. 2010; Suzuki et al. 2011; Inga Jácome et al. 2016; Guloksuz et al. 2017; Xie et al. 2017; Hu et al. 2018; Eftekharian et al. 2018; Carpita et al. 2020a), although these results were not always replicated (Al-Ayadhi 2005; Molloy et al. 2006; Ashwood et al. 2011a; Brocker et al. 2010; Suzuki et al. 2011; Inga Jácome et al. 2016; Guloksuz et al. 2017; Xie et al. 2017; Siniscalco et al. 2018; Carpita et al. 2020a). Several meta-analyses were conducted on this subject. Masi et al. (2015) conducted a meta-analysis on 17 previous researches about cytokine levels in ASD, measured in plasma or serum. They evidenced the presence, in ASD subjects, of increased peripheral levels of some chemokines linked to inflammatory cell recruitment from bloodstream to inflammatory sites, such as MCP-1, eotaxin, IL-8, as well as other cytokines mainly considered pro-inflammatory, such as IFN-γ, IL-1β and IL-6. They found instead lower levels of TGF-β. These authors failed to find differences between ASD subjects and controls for G-CSF, macrophage inflammatory proteins (MIP-1α and MIP-1β), CCL5, TNF-α, receptor antagonist IL-1Ra, IL-4, IL-10, IL-12p40, IL-17, IL-23, IL-1α. On the other hand, the authors themselves stressed the heterogeneity in the analyzed data. Among the parameters found altered in the reported meta-analysis, IFN-γ is involved in several functions. It is able to modulate the transition from innate to adaptive immunity and to enhance the sensitivity and the response of the immune system towards pathogens. It is produced by T cells and promotes the Th1 response against intracellular pathogens, through mechanisms of cytotoxicity and macrophage activation, while inhibiting the Th2 response, which produces also anti-inflammatory cytokines such as IL-10 (Masi et al. 2015). TGF-β1 was instead reported to have a strong immunosuppressant effect, playing a crucial role for immune system homeostasis, and also to promote cell proliferation, differentiation and growth (Masi et al. 2015). IL-1β is a cytokine with a great pro-inflammatory potential and has been often found increased in acute or chronic inflammatory processes. As reported above, it is also known to affect the hypothalamic-pituitary-adrenal axis and the hypothalamus, being involved in fever and sickness behavior response (Masi et al. 2015). More recently, Saghazadeh et al. (2019a; 2019b) confirmed in other two meta-analyses increased levels of pro-inflammatory cytokines IFN-γ, IL-1β, IL-6, and TNF-α in ASD patients (although MCP-1 and eotaxin were not included), together with reduced levels of the anti-inflammatory ones IL-10 and IL-1Ra. TNF-α is a cytokine involved in the innate immune response and principally produced

by macrophages. Peripheral and circulating TNF-α can pass across the BBB, as IL-1 and IL-6, exerting directly an effect on the CNS, while also microglia cells seem to secrete it. TNF-α is reported to act as a neurogenesis modulator, and increased levels of this cytokine may alter the permeability of both the BBB and the intestinal epithelial barrier (Saghazadeh et al. 2019a; Carpita et al. 2018; Carpita et al. 2020a). Noticeably, pre-eclampsia was reported to be associated in humans with increased TNF-α levels and ASD incidence (Saghazadeh et al. 2019a; Carpita et al. 2018).

During the last year, Zhao et al. (2021) conducted a wide meta-analysis on 63 studies, including also cytokines for which fewer researches were available, and reported different levels between ASD patients and controls for IL-6, IL-1β, IL-12p70, macrophage migration inhibitory (MIF), a promoter of the expression of pro-inflammatory cytokines in acute and chronic inflammatory processes, eotaxin-1, MCP-1, IL-8, IL-7, IL-2, IL-12, TNF-α, IL-17, and IL-4, although for some parameters the results varied depending on the measurement in plasma or serum.

Some studies conducted in ASD children further deepen the investigation, reporting an association between the severity of ASD symptoms, such as impaired communication and aberrant behavior, and plasma concentrations of MCP-1, RANTES (regulated upon activation, normal T cell expressed and presumably secreted), eotaxin, IL-1β, IL-6, IL-8 and IL12p40, all increased in ASD patients when compared with unrelated controls (Ashwood et al. 2011a; 2011b; Carpita et al. 2020a).

It was also reported that the pattern of altered cytokine levels in ASD may vary depending on the severity of the disorder. IL-4 levels were reported to be specifically correlated with severe ASD, although not all the studies found significant differences between ASD children and controls for this cytokine (Krakowiak et al. 2017; Siniscalco et al. 2018; Carpita et al. 2020a). On the other hand, an increase of IL-1β, IL-5, IL-8, IL-12p70, IL-13 and IL-17 was reported among subjects with high functioning ASD (Suzuki et al. 2011; Carpita et al. 2020a). A study highlighted increased levels of IL-12p70 among subjects with milder forms of ASD when compared with controls, while finding increased levels of IL12p40 and TNF-α in subjects with a greater severity of the disorder (Inga Jácome et al. 2016; Carpita et al. 2020a): these cytokines have been associated with severe ASD also by another author (Xie et al. 2017; Carpita et al. 2020a).

The role of cytokines in ASD was also investigated through mRNA expression studies: a reduced expression of IL-2 mRNA was found in ASD children, while TNF-α, IL-17, IL-6 mRNAs were reported to have an increased expression (Eftekharian et al. 2018). Studies on post-mortem ASD brain specimens have found the presence of neuroinflammation in samples obtained from patients of different age (Vargas et al. 2005; Morgan et al. 2010; Carpita et al. 2020a). In particular, microglia activation was often observed, as also highlighted in other psychiatric disorders (Vargas et al. 2005; Morgan et al. 2010; Onore et al. 2012; Bjorklund et al. 2016; Carpita et al. 2020a). Microglia is known to play a crucial role in shaping and maintaining synaptogenesis, sustaining CNS plasticity, but it also functions as a regulator of the immune response in the CNS, promoting, among other actions, tissue injury repair (Graeber and Streit 2010; Wake et al. 2011; Carpita et al. 2020a). In both rodent and human models, activated microglia has been often associated with cognitive and behavioral impairment, and some authors have reported an activation of microglia after psychological stress in animal models (Tynan et al. 2010; Fontainhas et al. 2011; Suguma et al. 2011; Giovanoli et al. 2013; 2015; Calcia et al. 2016; Bloomfield et al. 2016; Bollinger et al. 2016; Carpita et al. 2020a). Increased concentrations of TNF-α, IL-1β and IL-17 have also been found in the brain of ASD patients, whereas other investigations observed an increased IL-6 expression in this tissue (Wei et al. 2012a; 2012b; 2013; Carpita et al. 2020a).

Further researches investigated instead the effects in ASD patients of immunomodulatory and anti-inflammatory drugs, such as oral/intravenous immunoglobulins or corticosteroids, spironolactone, pentoxifylline, celecoxib or sulforaphane (Niederhofer et al. 2003; Mordekar et al. 2009; Handen et al. 2009; Duffy et al. 2014; Young et al. 2016; Nazimek et al. 2017; Marchezan et al. 2018; Carpita et al. 2020a). However, studies in this field are still limited and heterogeneous, so that, although some evidence of beneficial effects on ASD symptoms was reported, additional research is warranted to actually clarify the potential role of these therapeutic strategies (Marchezan et al. 2018; Carpita et al. 2020a). Noticeably, antipsychotics or antidepressants were reported, in turn, to have immuno-modulatory or anti-inflammatory properties, suggesting a reciprocal influence between CNS and the immune system (Nazimek et al. 2017; Carpita et al. 2020a).

As for other biological parameters, despite the relatively large amount of research focused on cytokine levels in ASD children, little attention was provided to ASD adults: few studies in the literature focused on them. Chroonenberghs et al. (2002b) investigated a sample of post-puberal ASD patients (age range = 12-18 years) and controls, by measuring whole blood IL-6, IL-10, IL-1 receptor antagonist (IL-1Ra), IFN-α and TNF- α as well as serum IL-6, IL-2 receptor and IL-1Ra. They reported only a higher presence of IFN-α and IL-1Ra in whole blood, together with a trend towards increased levels of IL-6 and TNF-α. Other authors reported reduced TGF-β levels in serum of young adults with ASD (n = 19; mean age = 3.4±2.6 years) when compared with 21 unrelated controls (mean age = 22.7±2.3 years) (Okada et al. 2007). Another study evaluated serum IL-1β, IL-6, IL-10 levels as markers of immune-inflammatory activation, together with endotoxin levels in 22 adult patients with severe ASD (mean age: 28.1±7.7 years) and 28 age matched controls mean age = 28.7±8.1 years) (Emanuele et al. 2010). They found significantly increased levels of endotoxin in the ASD group, which seemed also to be inversely correlated with the gravity of social impairment. Moreover, a non-significant trend towards increased levels of IL-1β and IL-6 was reported in the patient group (Emanuele et al. 2010).

Another poorly addressed issue is the investigation of inflammatory patterns among subjects with BAP or sub-threshold autistic traits: relatives of ASD children were used in some cases as a control group (Manzardo et al. 2012; Napolioni et al. 2013; Carpita et al. 2020a). One of these studies, evaluating 29 different cytokines, found significantly lower plasma levels of IL1α, IL-6, G-CSF, epidermal growth factor (EGF), fractalkine, MCP-3, MIP-1α, and MIP-1β in ASD children vs. unrelated healthy siblings of other ASD patients (Manzardo et al. 2012), while another study did not find differences in plasma levels for any of the 40 cytokines measured between ASD patients and their typically developed children (Napolioni et al. 2013). Studies focused on adult parents of ASD patients were mostly focused on evaluating cytokine levels in pregnant mothers of ASD probands (Jones et al. 2017; Carpita et al. 2020a). Globally, in this field no study properly investigated the differences in cytokine levels between ASD adults and their adult first-degree relatives (Carpita et al. 2020a).

In the framework of possible interactions between the immune system and neurodevelopment, it should be also considered that, as in the case of TRP metabolism, the immune system shows tight interplays with the gut microbiota. Since several

alterations in the microbiota composition, together with the presence of gastrointestinal symptoms, have been reported in ASD children, it was hypothesized that the immune system may be one of the pathways through which microbiota can communicate with the brain, thus exerting a central role in the "gut-brain axis" and putting one more factor into the equation (Carpita et al. 2020a). Data resulting from investigations conducted in rodent models suggest that microbiota may exert an activating effect on microglia depending on its composition, eventually leading to the increase of pro-inflammatory cytokines in the brain (Erny et al. 2015; Carpita et al. 2020a). It should be noted that, although several hypotheses were made about the possible impact of an altered immune system activation on brain biochemistry and functions, it is also possible that CNS and immune system impairment (and, eventually, gut microbiota alterations as well) would be underlain by the same biological mechanisms, including shared genetic underpinnings. Further research is needed to shed light on the interconnections between central and peripheral systems and on their role in psychiatric disorders, including ASD. It is not known if immune system and metabolic pathway deregulation should be considered, with respect to neurodevelopmental disorders, either as a consequence, a cause or a parallel condition. However, during early life, the CNS and immune system are supposed to develop in parallel, influencing each other: thus, possibly, these systems should be better conceptualized within an integrative perspective, in the framework of a multidirectional cooperation between the center and the periphery in shaping human pathophysiology (Carpita et al. 2020a).

1.5 BDNF in ASD

BDNF is a member of the neurotrophin family, a group of secreted proteins involved in a wide set of activities linked to development, function and survival of central and peripheral neurons. Besides BDNF, the neurotrophin family includes the Nerve growth factor (NGF), neurotrophin 3 (NT-3) and neurotrophin 4/5 (NT-4/5). BDNF was originally highlighted as a factor able to promote the peripheral neuron survival, but it is now known to be the most common and capillary distributed neurotrophin in the CNS (Martinowich and Lu 2008). BDNF seems to be able to regulate several crucial functions for the differentiation, survival, migration of neurons, as well as synapse

formation, growth and guidance of axons and dendrites. BDNF also regulates synaptic plasticity in adult life. In current available literature, its role in cognitive functions related to memory consolidation and acquisition has been widely stressed. During neurodevelopment BNDF seems also to support dopaminergic neurons differentiation and survival (Martinowich and Lu 2008; Francis et al. 2018). Moreover, BDNF was reported to promote the survival and regulate the development of 5-HT neurons, while 5-HT seems to upregulate BDNF expression, in a loop which may be involved in the efficacy of antidepressant therapies according to several authors (Martinowich and Lu 2008).

BDNF is a small homodimeric protein, encoded by the *BDNF* gene, showing multiple promoters, a complex regulation activity and different possible transcripts (Cattaneo et al. 2016). The BDNF synthesis process initially leads to its precursor, the pre-pro-BDNF protein. Subsequently, the pre-pro-BDNF pre-domain of 18 amino acid is cut and pro-BDNF is produced and transported in the cis-Golgi and then in the trans-Golgi, where it is packaged into secretory vesicles and then secreted following the constitutive pathway (when it is spontaneously secreted) or the regulated one (when it is secreted after stimuli) (Martinowich and Lu 2008; Cattaneo et al. 2016) (see **figure I7**). Most of the BDNF is secreted following the regulated pathway (Martinowich and Lu 2008). Before the secretion, the pro-BDNF is further cut to reach the mature form; however, it can also be secreted and then cleaved in the extracellular environment. Both BDNF and pro-BDNF are available in the CNS and their balance is crucial for synaptic plasticity. They bind to different receptors, exerting opposite effects: the pro-BDNF inhibits synaptic transmission and promotes synapsis elimination and axonal retraction (Michalski and Fahnestock 2003; Sun et al. 2012). The specific receptor for BDNF binding is the TrkB one, while the pro-BDNF binds the $p75^{NTR}$ receptor: this latter activates different sets of signaling pathways, including apoptotic ones. In physiological conditions, this effect is balanced by the anti-apoptotic signal derived from the TrkB receptor, in a synergic action which may be lost in pathological conditions (Hamanoue et al. 1999; Martinowich and Lu. 2008; Cattaneo et al. 2016). The activation of the $p75^{NTR}$ receptor in the hippocampus was reported to be linked to apoptotic signaling and NMDA receptor-dependent synaptic depression (Martinowich and Lu 2008).

It was reported that some cytokines may affect the balance between BDNF and pro-BDNF actions: TGF-β may up-regulate the transcription of BDNF and its TrkB receptor, while IL-1β would inhibit BDNF action (Sometani et al. 2001; Tong et al. 2012). Moreover, inflammatory processes may also affect BDNF production (Serrà-Milas 2016).

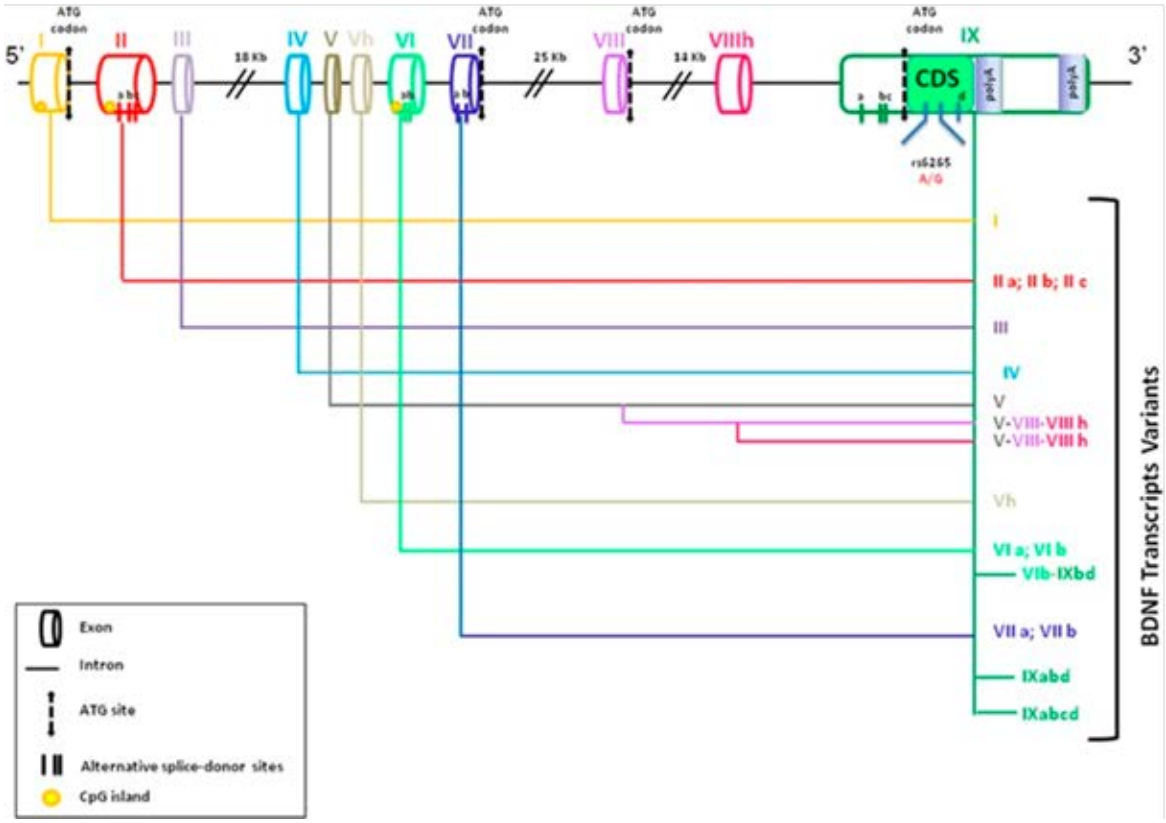

Figure I7. Structure of the human BDNF gene. The BDNF gene contains 11 exons (I–IX, Vh, VIIIh), which may combine in different transcripts. The Coding DNA sequence is in exon IX. Alternative splice-donor sites and CpG islands are also reported. DNA methylation may occur in association of CpG dinucleotides, or CpG islands, adding methyl groups to cytosine residues. CpG islands usually cluster around promoter regions, thus methylation may regulate the transcription of BDNF forms (from Cattaneo et al. 2016).

In the CNS, BDNF can be secreted in response to neuronal activity, deriving from pre- or post-synaptic sites: it was reported to regulate neurogenesis also in adults, and to

show a crucial role in hippocampal plasticity, being involved in memory functions, learning and long-term potentiation (LTP) mechanisms (Schinder et al. 2002; Martinowich and Lu 2008) (see **figure I8**). BDNF is mainly localized in neurons, but it can be also expressed by glial cells, in particular under metabolic stress (Nakajima et al. 1998). Besides hippocampus, the highest levels of BDNF in the CNS were reported in the neocortex, striatum, pro-encephalic nuclei, cerebellum and hypothalamus (Murer et al. 2001). In both the CNS and periphery, BDNF seems to be involved in cellular repair after injuries (Meeker and Williams 2015). BDNF may also mediate the action promoted by some neuropsychiatric drugs, such as antidepressants and lithium, on hippocampus neurogenesis: an effect that did not seem to be elicited by the same drugs in mouse models bearing defects of BDNF signaling (Sairanen et al. 2005; Martinowich and Lu 2008). Lower levels of BDNF were reported in several neurological and psychiatric disorders, and in particular mood disorders, Schizophrenia, or Alzheimer's disease (Molendjik et al. 2013; Ahmed et al. 2015; Fernandes et al. 2015inArmeanu; Ormstad et al), while stress-induced depressive behaviors have been found related to reduced BDNF levels in the hippocampus (Duman and Monteggia 2006; Martinowich and Lu 2008). On the other hand, in other pathological conditions, such as allergic diseases (e.g. asthma, urticaria, atopic dermatitis) increased BDNF levels were reported (Raap et al. 2005; Rössing et al. 2011; Prakash et al. 2014; Francis et al. 2018). In this framework, it should be noted that pro-inflammatory cytokines seem to be able to reduce the gene expression of BDNF and that of other neurotrophins (Calabrese et al. 2014). It was hypothesized that an impaired BDNF signaling may constitute a vulnerability factor for psychiatric disorders, affecting neuroplasticity, hypothalamic–pituitary–adrenal axis but also inflammatory processes (Cattaneo et al. 2016). On the other hand, it is also possible that inflammation may contribute to the pathogenesis of mood disorders through the reduction of BDNF, and that, more generally, BDNF may be the "bridge between inflammation and neuroplasticity" (Calabrese et al. 2014). Genetic studies have also demonstrated an association between the detection of point mutation allelic variants (single nucleotide polymorphisms, SNPs) of BDNF or TrkB genes and mood disorders, Schizophrenia, Obsessive-compulsive disorder and ASD (Fanous et al. 2004; Nishimura et al. 2007; Alonso et al. 2008; Correia et al. 2010).

In peripheral districts and in the bloodstream, most of the circulating BDNF is transported by platelets (Martinowich and Lu 2008). Several peripheral tissues, such as

muscles, including heart and vascular smooth muscle cells, but also lung, spleen or liver, express BDNF and TrkB mRNA; Moreover, BDNF can be produced by monocytes and lymphocytes (Nakahashi et al. 2000; Edling et al. 2004). BDNF was reported to pass through the BBB, and it was supposed by several authors that its circulating levels may reflect those in the CNS (Numakawa et al. 2010).

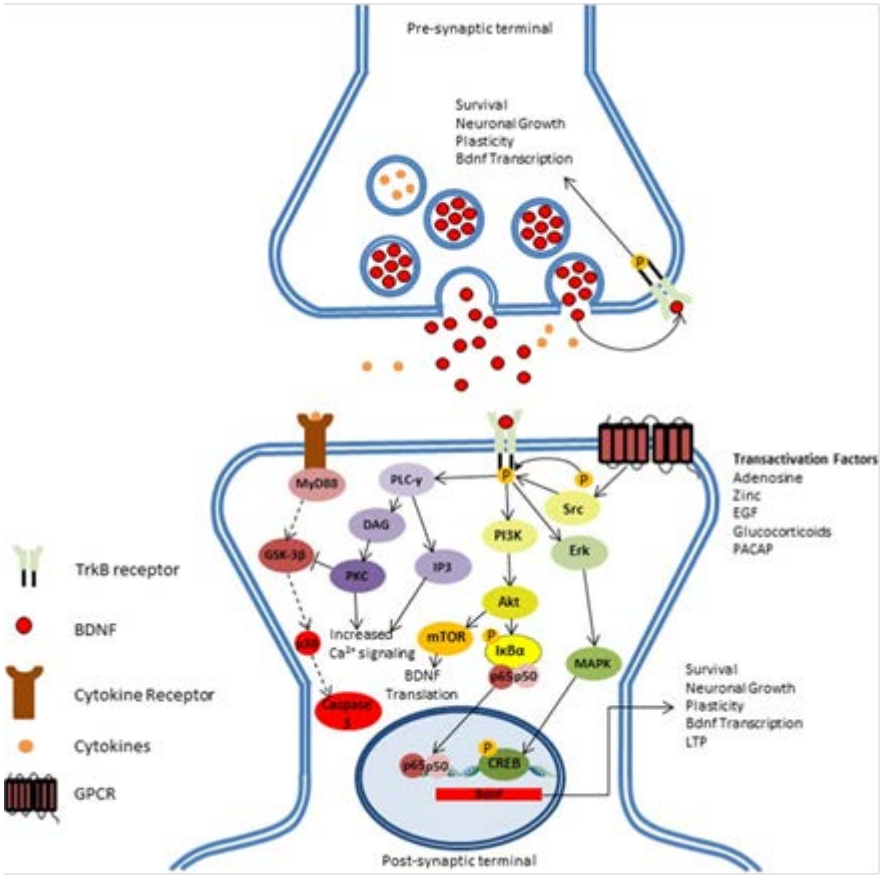

Figure I8. BDNF signaling. BDNF Binding to TrkB receptor may elicit different downstream pathways. Through the induction of phosphatidylinositol 3-Phosphate (PI3K), It may promote the transcription of BDNF mRNA by activating mTOR-dependent BDNF translation. BDNF can also modulate gene regulation through the induction of Akt and Erk downstream pathways and the activation of NF-kb and CREB transcription factors, respectively. Gene modulation promotes long-term potentiation, neuronal survival and growth, de-novo BDNF expression. Phospholipase C-gamma (PLC-γ) can induce short-term signaling through the increase of Ca2+ neuronal response, and, through inhibition of glycogen synthase kinase 3-beta (GSK-3β), can inhibit the inflammatory dependent apoptosis downstream (represented in dashed line) (From Lima Giacobbo et al. 2019).

In the field of ASD, many studies evaluated circulating BDNF levels in both plasma and serum, assuming that they were correlated to BDNF levels in the brain. Results from these studies are contrasting: while many authors reported increased BDNF levels in ASD, others did not find differences between patients and controls, while further researches reported decreased BDNF levels in ASD patients (Quin et al. 2016; Ormstad et al. 2018).

It was hypothesized that BDNF signaling alterations in the CNS may be responsible for the brain growth acceleration and increased brain size reported in ASD children, together with connectivity alterations (Billeci et al. 2016; Francis et al. 2018). Elevated BDNF levels may be also in line with some features reported in ASD, such as the enhanced synaptic plasticity and dendritic spine density, the increased protein synthesis at synapses, or also with the hypothesis of an increased BDNF production mediated by the enhanced sensory sensitivity (Armeanu et al. 2017). On the other hand, lower BDNF levels may be in line with the increased inflammatory vulnerability and altered cortical neurogenesis reported in some patients with ASD (Katoh-Semba et al. 2007; Lotrich et al. 2013; Francis et al. 2018). On the basis of the studies which had reported increased BDNF levels in ASD, two hypotheses were proposed on the possible role of BDNF in this condition. It would be possible that BDNF mediates a neural mechanism of the disease, in particular through promoting excitatory synaptic activity, which in physiological conditions would be negatively regulated by the activity of other receptors. Altered gene expression of some of these receptors has been indeed linked to other neurodevelopmental disorders, such as fragile X syndrome, which also features an increased risk of developing ASD. Increased BDNF levels may thus contribute to synaptic dysfunction, which might be involved in ASD pathogenesis or progression (Saghazadeh and Rezaei 2017). According to the other hypothesis, increased BDNF levels in ASD may instead constitute a compensatory mechanism promoting neuroplasticity. In this framework, we should mention the role of BDNF in mediating the improvement of cognitive performance after exercise or, more generally, the activity-dependent neuroplasticity, which was stressed by several authors (Martinowich et al. 2003; Saghazadeh and Rezaei 2017).

It is worth noting that, while most of the literature seems to point out the presence of increased BDNF levels in ASD children, despite several limits and the high

heterogeneity of the available research, other meta-analyses have instead shown lower levels of BDNF in mood disorders and Schizophrenia (Molendjik et al. 2014; Ahmed et al. 2015; Fernandes et al. 2015; Armeanu et al. 2017). In this framework, Armeanu et al. (2017) reported that Prader-Willi syndrome, which often features psychotic symptoms, was also linked to reduced levels of BDNF, while in the Angelman syndrome, which is characterized by opposite genetic alterations and a frequent comorbidity with ASD, BDNF levels were reported to be increased. The authors further highlighted how Schizophrenia, in contrast with ASD, seems to be associated with deficits in sensory sensitivity or also with reduction of dendritic spine density (Armeanu et al. 2017).

Recently several meta-analyses were published on this subject, and all globally reported higher BDNF levels in ASD patients than in controls, although high heterogeneity among the available studies was observed. Several possible factors may underlie these differences, including different methodologies in sample collection or preparation, different kinds of biochemical assays and diagnostic methods, differences in sample characteristics (age, specific form and severity of ASD, comorbid conditions, etc.). All the authors of recent meta-analytic studies stated that further researches on BDNF levels in ASD, employing more standardized protocols, would be necessary in order to clarify the issue (Armeanu et al. 2017). It should be noted that other studies independently reported that BDNF may vary depending on sex, age or weight also in the general population (Lommatzsch et al. 2005; Iughetti et al. 2011; Pillai et al. 2012; Spratt et al. 2015; Armeanu et al. 2017). Qin et al. (2016) performed a meta-analysis on 19 studies (1411 children, 1485 controls), excluding researches in adult samples, and found increased peripheral BDNF levels in ASD children. They included 4 studies which analyzed spot samples of dried blood from neonates, while the others were studies conducted on non-neonate ASD children analyzing BDNF levels in serum or plasma. When performing a further meta-analysis in the sub-groups, the authors reported increased BDNF levels only in the non-neonate ASD children when compared with controls. Moreover, a further analysis showed higher BDNF levels in ASD patients than in controls in studies on serum samples (n = 10) but not in those performed on plasma samples (n = 5). However, as already reported, high levels of heterogeneity among studies were found considering the whole sample as well as all the sub-groups (Quin et al. 2016). Zheng et al. (2016) performed instead a meta-analytic review on 14 studies for a total of 2707 participants, employing serum or plasma samples. They also included

available studies on adults, with a final mean age ranging from 0 to 22.2±2.2 years among studies. Besides reporting increased BDNF levels in ASD patients in the overall sample, they also confirmed this result in studies conducted on both serum (n = 13) and plasma (n = 1) samples. Similar results were reported when grouping studies depending on the diagnosis: ASD (n = 5) or Autistic disorder (n = 9). When considering the impact of the analytic procedures adopted, the authors reported significantly higher BDNF levels in ASD patients among those studies performed by means of enzyme-linked immunosorbent assay (ELISA) (n = 11) and recycling immunoaffinity chromatography (n = 1), but not among studies using Luminex (n = 3). Remarkably, they also reported that BDNF levels were significantly increased in ASD when analyzing studies on children (n = 10), without revealing significant differences in studies performed on adults (n = 4). They hypothesized that BDNF may be increased in ASD only during early life, in line with ASD animal models which also reported increased BDNF level in the foetal brain, and that this index may eventually underlie ASD pathogenesis or, alternatively, represent a compensatory response to the late brain maturation (Zheng et al. 2016). Saghazadeh and Rezaei (2017) selected for their meta-analysis 18 studies, including a total of 1788 participants, both adults and children, reporting increased BDNF levels among ASD patients in the whole sample but not when separately analyzing studies conducted on different biological matrices: plasma (n = 4), serum (n = 11) or dried newborn bloodspots (n = 3). They also reported no significant differences in BDNF RNA expression between patients and controls, on the basis of three main studies (Saghazadeh and Rezaei 2017). Armeanu et al. (2017) in their meta-analysis evaluated a sample including 1242 participants obtained from 15 studies, reporting increased BDNF levels in ASD patients. As in the case of previously mentioned authors, they reported a high heterogeneity among the investigations available in the literature, although stating that their results were not relevantly affected by the type of biological specimen evaluated (serum, n = 12, or plasma, n = 3).

Some BDNF studies have also focused on BAP. For instance, Francis et al. (2018) also included in their investigation a group of parents of ASD probands. They recruited a sample of young children with ASD (n = 45) without intellectual disability or allergies and with an age range of 30-42 months. They found significantly lower BDNF serum levels in ASD patients than in controls of the same age range (n = 26). They hypothesized that their findings could be in contrast with respect to most of previous

literature due to the lack of intellectual disability, in line with other studies that had previously revealed negative correlations between intelligence and BDNF amounts or a link between intellectual disability and increased BDNF (Yeom et al. 2016; Francis et al. 2018; Ormstad et al. 2018; see also paragraph **1.3**). Moreover, they did not find any correlation between BDNF and ASD symptoms, underlining that this result was in accordance with previous researches, except for two studies (Zhang et al. 2014; Meng et al. 2017; Francis et al. 2018). On the other hand, they reported higher BDNF levels in parents of ASD children (n = 82) than in control parents (n = 44). They did not find any relationship between BDNF and autistic traits in parents, although they reported that BDNF levels in parents correlated with those measured in their children, a finding positively influenced by the presence of BAP features when considering the father group, although the correlation was instead weakened by the presence of BAP features in mothers. Another study published in the same year (Brondino et al. 2018) evaluated also the correlation of circulating BDNF, vasopressin and OT levels with autistic traits, measured by the Autism spectrum quotient (AQ) in a non-affected population of students and faculty staff members. Their results highlighted that, among all the considered variables, BDNF levels only were found to positively predict the presence of autistic traits. This led the authors to hypothesize a possible continuum of BDNF level alterations in the autism spectrum.

1.6 Markers of altered methylation and trans-sulfuration processes in ASD

In the context of the search and appraise of possible metabolic signatures of ASD substrates of the transmethylation metabolism of the sulfur-containing amino acid methionine such as S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH) and homocysteine (HCY), are acquiring growing interest (Melnyk et al. 2012): these metabolites are in fact signals of methyltransferase activity and methylation, indicating specific patterns of methylated substrates, including DNA, and gene transcription state (Palego et al. 2015a; 2015b). Trans-sulfuration of methionine leads instead to cysteine and its derivatives (glutathione), endowed with powerful antioxidant properties, which have been found reduced in autistic patients (Chauhan and Chauhan 2006). Among various biological/metabolic parameters, HCY was supposed to be involved in the

pathophysiology of several kinds of disorders, including neuropsychiatric conditions and ASD, but also stroke and thrombosis (Ali et al. 2011). It seems to be also a potential biomarker in neurodegenerative diseases of the elderly, such as Alzheimer's disease, for which increased HCY levels were considered both a possible risk factor and a predictive element that may be present even years before the onset of symptoms (Hermann and Obeid 2011; Ali et al. 2011). Higher levels of HCY were also reported in mood disorders and schizophrenia (Dittman et al. 2008; Kale et al. 2010; Han et al. 2015). HCY might directly affect behavioral and cognitive features, even in the general population: an inverse correlation has been reported between HCY levels, cognitive performance and hippocampal volume, while alterations of this latter have also been observed in ASD children (Budge et al. 2002; Barnea-Goraly et al. 2014; Han et al. 2015).

Elevated or accumulated HCY can exert excitotoxic effects, by reducing mitochondrial energy production and increasing excitotoxic effects of neurotoxic heavy metals, such as lead or mercury, or the neuronal vulnerability towards oxidative/excitotoxic injuries (Blaylock 2009; Ali et al. 2011). HCY seems to overstimulate NMDA receptors and potentiate glutamate excitotoxicity (Ho et al. 2002). It can impair redox homeostasis of both neurons and astrocytes (Zou and Banerjee 2005; Loureiro et al. 2010; Han et al. 2015). In particular, products and substrates such as HCY-thiolactone, N-HCY-protein, and Nepsilon-HCY-Lys, all derived from protein-related HCY metabolism, can damage proteins and induce structural changes, generating toxic and autoimmunity-triggering proteins (Jakubowski and Glowacki 2011; Han et al. 2015; Fu et al. 2018) (see also **figure I9**).

HCY is produced by demethylation of dietary methionine and it can be transformed into cysteine through trans-sulfuration processes, or re-methylated to methionine, through folate-dependent HCY methylation reactions (Ali et al. 2011; Zheng et al. 2017). High, potentially toxic levels of HCY are transformed into methionine by the methionine synthase (MS) enzyme. Methylenetetrahydrofolate reductase (MTHFR), which modulates also folate availability, is a key enzyme for the passage from methyl group transfer reactions to the synthesis of nucleotides: altered concentrations of MTHFR directly affect the DNA synthesis (James et al. 2008; Ali et al. 2011).

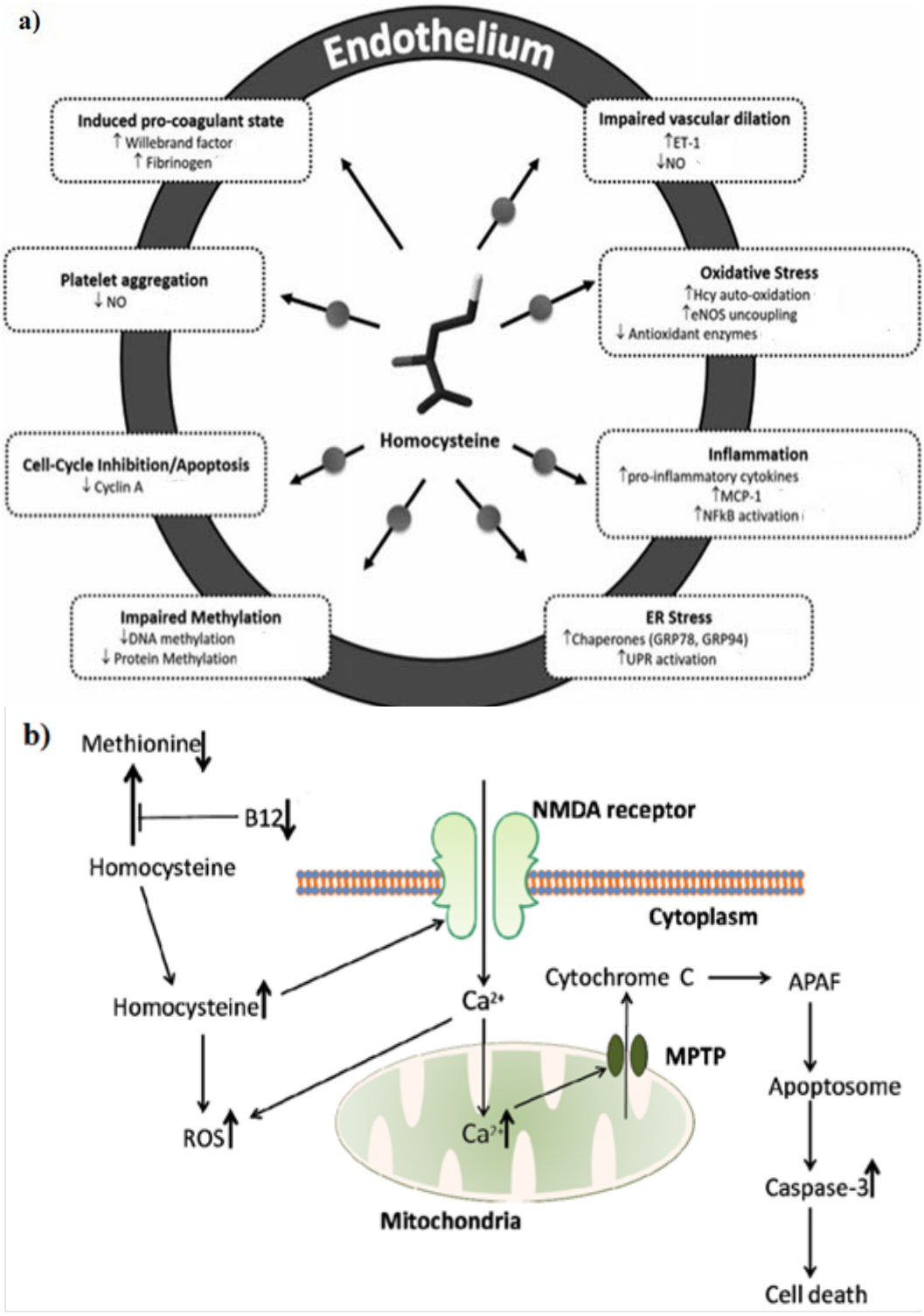

Figure I9. Schematic representation of HCY harmful effects. a) The endothelium: Arrows with dots indicate that these effects are associated also with SAH increase and hypomethylation (From Barroso et al. 2019). b) Cell-death induced by hyper-HCY. HCY may increase reactive oxygen species (ROS), eventually leading to apoptotic cell death. Moreover, NMDA receptor activation by HCY may also lead to ROS increase through an influx of calcium into the cell. eNOS: endothelial nitric oxide synthase;

ET-1, endothelin 1; GPx1, glutathione peroxidase 1; GRP78, glucose-regulated protein 78; GRP94, glucose-regulated protein 94; HCY, homocysteine; IL6, interleukin 6; IL8, interleukin 8; MCP-1,(monocyte chemoattractant protein 1; NF-κB, nuclear factor-kappa B; NO, nitric oxide; SAH, S-Adenosylhomocysteine; SOD, superoxide dismutase; UPR, unfolded protein response (adapted from Savage and Ma 2014).

It was reported that reduced MS activity and increased levels of the *MTHFR* allele variant C677T may lead to increased HCY levels: of note, in the specific case of autism, no association between the presence of *MTHFR C6777T* allele and a higher risk for ASD was found (dos Santos et al. 2010; Ali et al. 2011). On the other hand, it should be noted that SAM, a source of HCY and the main donor of methyl group in DNA methylation, may provide information on cell methylation capacities, including those related to DNA (Ali et al. 2011).

Cellular methylation capacity may be expressed as the ratio between the methyl donor SAM and the methylation inhibitor SAH (or SAM/SAH), which was found reduced in ASD children (Melnyk et al. 2012). In this framework, HCY alterations gained much more interest, together with the increasing researches focused on the use of biomarkers of DNA-methylation in neuropsychiatric disorders (Levenson 2010; Ali et al. 2011). At the same time, also altered trans-sulfuration pathways may have relevance in this field, since this metabolic state can increase oxidative stress, mainly affecting glutathione synthesis (Han et al. 2015). Glutathione is a non-protein thiol, a tripeptide composed of glutamate, glycine and cysteine, with a crucial role as antioxidant specie through its neutralizing action towards reactive oxygen species: in particular, the antioxidant capacity is maintained by the glutathione thiol/disulfide redox couple (GSH/GSSG) (Melnyk et al. 2012; Han et al. 2015). The cystathionine β-synthase (CBS) permanently removes HCY from the methionine cycle, starting the trans-sulfuration process that will lead to the synthesis of physiologically relevant S-containing molecules, as cysteine, glutathione, sulfate and taurine. The limiting amino acid for the synthesis of glutathione is cysteine, a protein amino acid less concentrated in the body than glycine and glutamate (Finkelstein 1988; Han et al. 2015) (see **figure I10**).

Increased levels of HCY may be underlain not only by genetic factors but also by nutritional issues. HCY accumulation may be linked to an insufficient intake, altered absorption or metabolic use of vitamin B6, B12 and folate, since their levels activate HCY metabolism through both trans-sulfuration and methylation pathways (Ali et al. 2011; Han et al. 2015).

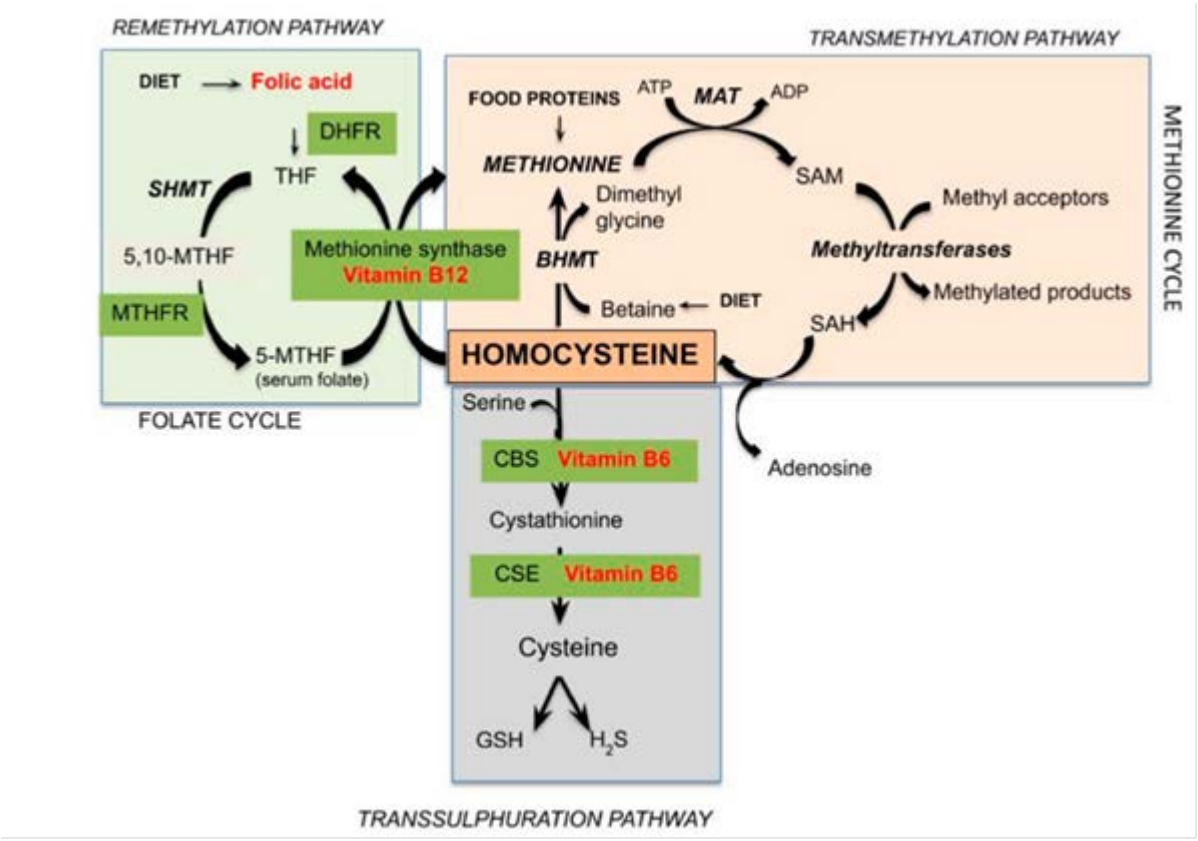

Figure I10. Schematic representation of the pathways of HCY-related metabolism. ADP: adenosine diphosphate; ATP: adenosine triphosphate; DHFR: dihydrofolate reductase; BHMT: betaine-HCY S-methyltransferase; CBS: cystathionine β-synthase; CSE: cystathionase; GSH: glutathione; H_2S: hydrogen sulphide; MAT: methionine adenosyltransferase; MTHF: methylenetetrahydrofolate; MTHFR: 5,10-methylene-THF reductase; SAH: S-adenosyl-HCY; SAM: S-adenosylmethionine; SHMT: serinehydroxymethyltransferase; THF: tetrahydrofolate (from Azzini et al. 2020).

An inverse correlation between the concentrations of these nutrients and HCY has been reported, while their intake seems to show efficacy in reducing HCY concentrations (Kałużna-Czaplińska et al. 2011a; 2011b; Ali et al. 2011). As reported above, an

increased prevalence of gastrointestinal problems, as well as altered eating patterns, such as selective food choice, potentially facilitating nutrient deficiency, has been found among ASD patients (Kałużna-Czaplińska et al. 2009; Ali et al. 2011; Carpita et al. 2020c). Reduced levels of folate and vitamin B12 have also been observed by some authors in ASD patients (Ali et al. 2011), although this finding was not always confirmed (Melnyk et al. 2012). On the other hand, some studies have highlighted beneficial effects of nutritional supplements on ASD symptoms, stressing the importance of diet in this population (Xia 2011; Ali et al. 2011). A higher oxidative stress condition was reported among ASD children, mainly associated with glutathione depletion and increased expression of SOD (Al-Mosalem et al. 2009; Ali et al. 2011).

Trans-sulfuration pathways were also reported to be impaired in ASD, with altered levels of HCY, cysteine, and glutathione (Ghanizadeh et al. 2012; Han et al. 2015). Globally, the trans-sulfuration and methylation pathways of HCY are crucial for the synthesis of DNA precursors, DNA methylation, subsequent regulation of gene expression by epigenetic mechanisms, and redox balance through glutathione synthesis. A correct balance of these systems is of the utmost importance for maintaining cell programming, in particular for shifting between proliferation, differentiation and apoptosis, during lifetime and especially during neurodevelopment (Melnyk et al. 2012).

In the field of ASD, the levels of HCY and associated metabolites have been evaluated only in children or, in fewer cases, in adolescents. One of the first studies in this research area was conducted by James et al. (2004), who reported significantly lower plasma levels of methionine, SAM, HCY, cystathionine, cysteine, glutathione and significantly higher levels of SAH, adenosine, and oxidized glutathione in ASD children (n = 20) than in controls (n = 33). These unbalances seem to be responsive to nutritional intervention trials with folic acid, betaine, and methylcobalamin. Globally, these authors highlighted the hypothesis of increased vulnerability towards oxidative stress and decreased methylation capacity in ASD. The observed reduction of methionine and SAM levels in ASD led the authors to assume that there was also a reduced activity of the MS enzyme, but the concomitant decrease of HCY levels in patients resulted discordant with this hypothesis, being quite difficult to explain (James et al. 2004). Thus, the authors further explained their results by rather suggesting an oxidative inactivation of MS in addition to the decrease of SAH hydrolase (SAHH) activity: this

latter aspect may be underlined by the report of increased adenosine, whose higher concentrations are known to reduce the activity of SAHH, through the binding on its active site. According to this model, which would also explain SAH increase and SAM decrease, HCY reduction would be explained by the diminished SAH hydrolysis linked to increased adenosine, consequently leading to a decreased HCY synthesis (James et al. 2004). A similar result about HCY was reported by Bala et al. (2016), whose data showed reduced levels of HCY, methionine, cystathionine, cysteine, vitamin B12 and vitamin 4 in 21 children and adolescents with ASD (age range = 2-18 years) than in 21 age and sex-matched controls. However, other studies reported an increase of HCY in ASD children in both plasma and serum (Ali et al. 2011; Tu et al. 2012; Cai et al. 2016; Wang et al. 2016; Guo et al. 2020; Zheng et al. 2017), as also firstly expected by James et al. (2004). Furthermore, there are works that did not find differences in HCY levels between ASD children and controls (Adams et al. 2007; Main et al. 2015). A recent meta-analysis by Guo et al. (2020), conducted on 31 studies, has found increased levels of both serum and plasma HCY in ASD children than in controls. These authors also evaluated possible differences depending on the ASD subtype, reporting higher HCY levels in both children diagnosed with Autistic disorder than in children diagnosed with the broader category of ASD. Some authors specifically focused on Asperger syndrome. Paşca et al. (2009) investigated the methionine cycle, the trans-sulfuration pathway, folate, vitamin B12 and the *C677T* polymorphism of the *MTHFR* gene in children with Autistic disorder (n = 15), Asperger syndrome (n = 5), PDD-NOS (n = 19) and controls (n = 25, sex and age matched). They reported lower plasma levels of methionine, α-amino butyrate in the Autistic disorder and PDD-NOS group, while cysteine and glutathione were found reduced only in the Autistic disorder group. No significant difference was found for HCY. Parellada et al. (2011) investigated instead the total antioxidant status, non-enzymatic (glutathione, HCY) and enzymatic (catalase, SOD, glutathione peroxidase) antioxidants as well as lipid peroxidation in adolescents with Asperger syndrome (n = 35; mean age = 12.89±2.58 years), adolescents with a first episode of psychosis (n = 34) and healthy controls (n = 34), reporting reduced total antioxidant status in Asperger syndrome and increased HCY levels in the psychotic patients. In urine samples, HCY levels were found mainly increased in ASD patients (Kałużna-Czaplińska et al. 2011a; 2011b; Ali et al. 2011; Noto et al. 2014; Puig-Alcaraz et al. 2015; Zheng et al. 2017), although this result was not confirmed by all the studies,

with some authors reporting instead decreased levels in this population (Liu et al. 2019). Intriguingly, Puig-Alcaraz et al. (2015) showed not only increased urinary levels of HCY in ASD children, but also a significant correlation between urinary HCY levels and impaired communication skills; on the other hand, no correlation was obtained with socialization deficits and repetitive/restricted behaviors as measured by the revised Autism diagnostic interview (ADI-R). Han et al. (2015), in a sample of 50 ASD children and 50 age- and sex-matched controls, evaluated a wide set of metabolites of trans-sulfuration metabolism, such as HCY, cysteine, total glutathione, reduced (GSH) and oxidized glutathione (GSSG), reporting in ASD children higher levels of HCY and GSSG, together with lower levels of cysteine, total glutathione, GSH and GSH/GSSG ratio. Moreover, they found a specific positive correlation between HCY and the score reported at the Childhood autism rating scale (CARS) in patients, while an inverse correlation was obtained between cysteine levels and the Autism behavior checklist (ABC) scores.

Some of these investigations also included relatives of ASD subjects. Melnyk et al. (2012) compared 68 ASD children, 40 unaffected siblings and 54 age-matched controls. They reported significantly lower levels of methionine, total cysteine, SAM and SAM/SAH ratio as well as a lower percentage of DNA 5-methylcytosine among ASD children when compared with siblings and controls. The methylation inhibitor SAH, as well as the oxidized cystine disulfide form of cysteine (Cys-SS), was found instead to be increased in the ASD group with respect to controls. Adenosine, another methylation inhibitor, was found increased in the ASD group with respect to siblings but not to controls. On the other hand, SAH levels were shown to be intermediate between ASD and controls in siblings, being significantly higher than in controls. Siblings showed also intermediate levels of free glutathione: lower than controls but higher than ASD group. No difference was found for HCY, folate and vitamin B12. Both the main extracellular and intracellular redox buffers (Cys/CySS and GSH/GSSG respectively) were found in a more oxidized state (reduced ratio) among ASD children. Main et al. (2015) compared genomic stability, by using the cytokinesis-block micronucleus cytome (CBMN-cyt) assay, B vitamins and HCY in ASD children (n = 35), their unaffected siblings (n = 27) and unrelated controls (n = 25). They reported a significant increase of vitamin B2 in the ASD and sibling group, while no significant differences

were reported for CBMB-cyt biomarkers, HCY and other B vitamins: these findings led the authors to hypothesize that genomic instability would not be a typical ASD feature.

Besides studies performed by appraising pregnant mothers (Chen et al. 2016), some authors also specifically reported increased HCY levels among parents of ASD probands. James et al. (2008) investigated the levels of HCY, methionine, cysteine, SAM, SAH, total glutathione, GSSG and GSH as well as 5-methylcytosine and total cytosine in DNA among 46 mothers and 40 fathers of ASD children (age range = 21-45 years) and 200 control mothers (age range = 17-43 years). Their results showed increased levels of HCY, SAH and GSSG, but lower GSH levels and GSH/GSSG or SAM/SAH ratio in parents of ASD children. Moreover, they found a hypo-methylated DNA, evaluated by the percent 5-methylcytosine/total cytosine, in parents with SAH levels > 30 μMol/L. In a further study conducted in a wider sample, the authors reported increased plasma levels of HCY, adenosine, and SAH among mothers of ASD patients when compared with control mothers, while methionine and folate levels, together with SAM/SAH and methionine/HCY ratio, were reduced (James et al. 2010). A significant hypomethylation state of the DNA in mothers of ASD children was also reported. Moreover, the authors highlighted a significantly higher presence of the reduced folate carrier (*RFC1*) *G* allele in mothers of ASD subjects, although it was not found in fathers or in children. The presence of maternal *G* allele, but not the child genotype, was associated with a significantly higher ASD risk. They did not find significant differences in the allele frequencies of *MTHFR C677T, MTHFR A1298C, TCII C776G,* or *MTRR A66G* polymorphisms between parents, children and controls.

Although studies on children in this field seem to point out altered methylation/trans-sulfuration pathways and increased levels of HCY in the autism spectrum, including in relatives of subjects with ASD, studies in this field are still limited to children, and further research should also investigate the presence of these patterns in ASD adults. This could be a specific and convergent point of interest, considering that redox status and epigenetic modifications may also be considered adaptive responses to environmental stressors, with eventual changes during lifetime (Melnyk et al. 2012; Han et al. 2015).

2. Aims of this study

As reported above, although several authors highlighted the presence of biochemical alterations in ASD, most of the studies were conducted on children, and there is still a lack of knowledge on possible biochemical correlates of ASD in adults, with a very limited number of studies focused on this specific population (Gabriele et al. 2014; Carpita et al. 2020). Improving our knowledge about possible biochemical correlates of ASD in adult samples is of particular interest, considering that recent literature has stressed the importance of focusing on adult forms of ASD, which often may remain under-diagnosed, in particular when not associated with intellectual or language impairment, but which nevertheless may deeply affect the quality of life, being also a specific risk factor for the development of other psychopathological conditions (Dell'Osso et al. 2019a; Carpita et al. 2020a). Moreover, the presence of intermediate autism spectrum phenotypes (showing traits similar, although sub-threshold, to those typically defining the full-fledged disorder) among first-degree relatives of ASD patients is well-known in the literature. These traits have been often associated with detrimental effects on quality of life and psychopathological risk (Carpita et al. 2020b). These evidences, as reported in the introduction section, led to conceptualize the presence of a BAP, which was also supported by neuroimaging studies showing in parents or siblings of ASD patients neurostructural and neurofunctional alterations similar, but milder, than those detected in the probands (Billeci et al. 2016). Despite that, limited attention was paid on the search of valuable biochemical correlates of BAP in relatives of ASD patients, who in some studies were instead included as controls (Carpita et al. 2020a).

In this framework, the aim of the present investigation was to evaluate potential biochemical markers and correlates of sub-threshold (BAP) and full-threshold ASD in adults without language or intellectual impairment, focusing on the biochemical parameters considered among the most promising for ASD in the currently available literature, as reviewed in the previous chapters. A biomarker has been defined as a objectively measurable characteristic that can be considered as an indicator of physiological or pathological processes, or of a response to a pharmacological intervention. Biomarkers can be used as diagnostic tools for identifying patients affected by a given condition as well as for predicting and monitoring the response to

therapeutic treatments (Biomarkers definitions working group 2001; Banati and Hickie 2009; Masi et al. 2017).

In particular, we aimed to appraise, among subjects with ASD (ASD group), their first-degree relatives (BAP group), and unrelated healthy controls (CTL group) the circulating concentrations of: 1) 5-HT, as the most investigated biochemical correlate in ASD children; 2) TRP, KYN, KYNA and QUIN, in order to evaluate the main TRP metabolic pathways; 3) BDNF, as a main synaptic modulator and peripheral inflammation mediator; 4) IL-6, as one of the most investigated and frequently reported altered cytokines in ASD children; 5) HCY, as a potential marker of impaired trans-sulfuration and trans-methylation processes in this population. Moreover, we aimed to evaluate eventual correlations between the amount of these biochemical parameters and autism spectrum symptoms/traits as measured by the psychometric scales most frequently employed in this field. Our results may shed light on possible biochemical correlates of autism spectrum symptoms, conceived as a psychopathological continuum, as well as on differences between sub-threshold and full-threshold ASD phenotypes. This study might also improve current knowledge on the potential biochemical mechanisms associated with autism spectrum pathophysiology, envisaged as integrative pathways and networks acting at the interplay between central and peripheral systems in shaping psychopathology (Carpita et al. 2020b). From a clinical point of view, results from this study may pave the way to future research focused on improving diagnostic procedures and tools, with the possible employment of biochemical markers, and, eventually, on identifying new potential treatment perspectives for ASD.

3. Methods

3.1 Recruitment procedures

For the aims of this project, we recruited a group of ASD subjects (ASD group), a group of their first-degree relatives (parents or siblings) (BAP group) and a control group (CTL group). The ASD group was recruited among adult out-patients or in-patients treated at the Psychiatric Unit of Pisa University Hospital. Subjects must have received a diagnosis of ASD during childhood or in adult life. The diagnosis was also clinically confirmed at the time of the recruitment by trained psychiatrists. Major exclusion criteria were: age below 18 or over 65 years; being unable to fulfill assessments due to language or intellectual impairment; presence of neurodegenerative diseases or other relevant medical or neurological disorders; a diagnosis of Schizophrenia or of substance use disorder. The BAP group was enrolled during the recruitment of the ASD group, by requesting the participation of one relative (a parent or a sibling) for each patient. Exclusion criteria were the same used for the ASD group, with the exception of age range: in the BAP group, subjects were excluded only if they had an age below 18 years. Additional exclusion criteria for this group were: having received a diagnosis of ASD according to DSM-5. CTLs were instead recruited on a voluntary basis. For this group, in addition to the exclusion criteria used for the ASD group, subjects were also excluded if they have received a diagnosis of a psychiatric disorder according to DSM-5. All the recruited subjects were assessed by means of a structured clinical interview and psychometric scales. A blood sample was also collected from all participants in order to perform the biochemical evaluations. All participants received clear information about the study and had the opportunity to ask questions before providing a written informed consent. All data were treated according to Italian and European Privacy laws and rules. The study was conducted in accordance with the declaration of Helsinki, and all procedures were approved by the local ethical committee.

3.2 Psychometric instruments

All the subjects were assessed by trained psychiatrists with the Structured Clinical Interview for DSM-5 disorders (SCID-5) in order to evaluate the presence of other psychiatric conditions. Moreover, they were assessed with psychometric questionnaires in order to evaluate the wide spectrum of sub-threshold and full-threshold autistic symptoms, as well as associated traits, and their impact on global functioning.

The Structured Clinical Interview for DSM-5 disorders (SCID -5)

The SCID-5 is the gold standard structured clinical interview for investigating the presence of major psychiatric disorders according to DSM-5 (First et al. 2015). It must be administered by trained mental health professionals. It is composed of 10 independent modules; the sequence of questions follows the order of the related diagnostic manual (DSM-5) and the different items of each module guide the interviewer through the evaluation of the presence of symptoms that may satisfy the diagnostic criteria.

The Adult Autism Sub-threshold Spectrum (AdAS Spectrum)

The AdAS Spectrum was developed and validated by Dell'Osso et al. (2017) in order to assess the broad range of lifetime sub-threshold and full-threshold manifestations related to the autism spectrum among adults without language or intellectual impairment. It is the first questionnaire developed on the basis of the DSM-5 criteria and description for ASD, and specific attention was paid on including items about the altered reactivity to sensory inputs as well as about possible gender-specific or atypical manifestations. It is composed of 160 dichotomous items (yes/no), divided in 7 domains: *Childhood/adolescence*, *Verbal communication*, *Non-verbal communication*, *Empathy*, *Inflexibility and adherence to routine*, *Restricted interests and rumination*, *Hyper-hypo reactivity to sensory input*. The instrument has 2 cut-off scores, 43 for the presence of sub-threshold autistic traits and 70 for the presence of ASD clinical symptoms (Dell'Osso et al. 2020a). For each domain and total score, higher scores are associated with more severe impairment. The validation study highlighted the excellent reliability of the questionnaire (Kuder-Richardson's coefficient = 0.964) and its strong correlation with other scales frequently used in this field, such as the Ritvo Autism and Asperger Diagnostic Scale, 14-item version (RAADS-14) (Pearson's r correlation = 0.83) or the AQ (Pearson's r correlation = 0.77) (Dell'Osso et al. 2017).

The Ritvo Autism and Asperger Diagnostic Scale (RAADS-14)

The RAADS-14, a brief version of the RAADS-revised, is a questionnaire composed of 14 items developed with the aim to concisely assess the presence of the main autism spectrum symptoms. Answers are organized in a 4-point Likert scale ("true now and when I was young", "true only now", "true only when I was younger than 16", "never true"), with scores ranging from 3 to 0 (higher scores associated with greater impairment). Items are grouped in 3 domains: *Mentalizing deficits* (which included items investigating awareness about difficulties in social interaction, language and circumscribed interests), *Social anxiety* and *Sensory reactivity*. In the validation study, the RAADS-14 showed excellent internal consistency, with a Crombach's alpha = 0.90. A cut-off score of 14 or above was also proven useful for identify subjects with ASD, with a good sensitivity but lower specificity (Eriksson et al. 2013).

The Autism Spectrum Quotient (AQ)

The AQ is one of the oldest questionnaires developed for measuring the whole spectrum of ASD symptoms in adults without intellectual impairment. It is structured in 50 items, with a 4-point Likert scale scoring system (answers ranging from "definitely agree" to "definitely disagree"). Higher scores suggest a greater presence of autistic symptoms. Items are grouped in 5 sub-scales: *Social skill*, *Attention switching*, *Attention to detail*, *Communication* and *Imagination*. The AQ showed good reliability measures, with moderate to high alpha coefficients for the five domains (Baron-Cohen et al. 2001). Different possible cut-off scores were proposed for this scale (Baron-Cohen et al. 2001; Wheelwright et al. 2010).

The Ruminative Response Scale (RRS)

The RRS is an instrument which explores the tendency towards ruminative thinking. Each item is rated on a 4-point Likert scale, with responses ranging from "almost never" to "almost always". The items are grouped for evaluating 3 specific dimensions: *Brooding*, *Reflection* and *Depression.* Higher scores are associated with a higher tendency to ruminative thinking. The scale was reported to have an excellent internal consistency, with Crombach's alpha = 0.89 (Nolen-Hoekema and Morrow 1991). In the present work, the 22 item version of the instrument was used (Palmieri et al. 2007).

The Work and Social Adjustment Scale (WSAS)

The WSAS is a 5-item questionnaire frequently used in the literature with the aim to evaluate the impact of symptoms on social and work functioning (ability to *Work*, *Home*

management, *Social leisure activities*, *Private leisure activities*, *Ability to form and maintain close relationships*). For each item, subjects are requested to indicate how much their symptoms impact on their "ability to carry out the activity", from "Not at all" to "Very severely", in a 9-point Likert scale (scores ranging from 0 to 40, with higher scores indicating a greater impairment). In the validation study, the instrument showed good internal consistency, with alpha coefficients ranging from 0.80 to 0.90 (Mundt et al. 2002).

3.3 Biochemical evaluations

3.3.1 Instruments, chemicals and reagents

All reagents and chemicals used for this work were of the best quality and purity according to laboratory standards. The water used for the preparation of all the required solution was ultrapure HPLC gradient-grade distilled milli-Q water, with 18 MΩ cm^{-1} resistivity, prepared by means of a Simplicity Millipore Apparatus coupled to an ultraviolet (UV) lamp and a 0.2-micron filter in order to prevent contamination by bacteria, other kinds of biological agents, or particles. A PST-60HL plate thermo-shaker (Biosan, Riga, Lativia) was used during ELISA procedures. For absorbance measuring, a 96-well plate spectrophotometer (Multiskan FC ThermoScientific, Thermofisher Scientific, Waltham, MA, USA) was employed. (see **figure M1**).

Figure M1. a) the multiskan spectrophotometer; b) the plate thermo-shaker.

3.3.2 Blood sampling, sample preparation and storage procedures

All blood samples were accurately handled avoiding blood hemolysis by trained and authorized nurse professionals of the "Azienda Ospedaliera Universitaria Pisana", Psychiatric Unit, Department of Clinical and Experimental Medicine. 15 mL of venous blood were withdrawn from all the subjects, who were requested to fast from the previous evening and at least for 12 hours. Blood withdrawals were scheduled between 9 and 10 a.m., in order to avoid circadian rhythm influences on the investigated parameters. Blood was collected in different vacutainer tubes, as follows: 9 mL were gathered in 3 vacutainer tubes containing tripotassium ethylenediaminetetraacetic acid (K_3EDTA) as the anticoagulant, for separating the PPP from platelets; 3 mL were collected in 1 vacutainer tube containing lithium-heparin for plasma separation; 3 mL were collected in 1 vacutainer tube without anticoagulant and with a clot activator for serum separation. Vacutainer tubes were then immediately transported in proper thermostatic containers to the Biochemical laboratory of the Department of Pharmacy, University of Pisa, for sample preparation procedures. For PPP and platelet preparation, the K_3EDTA vacutainer tubes were centrifuged within 30 minutes from withdrawal. All centrifugations were performed at room temperature (RT). The first centrifugation was conducted at low speed (150 g for 15 minutes) in order to separate the PRP from the other cellular elements (Betti et al. 2018). Subsequently, the PRP volume was measured and then transferred in 2 Falcon tubes (capacity = 15 mL) and centrifuged again, together with lithium heparin and clot activator tubes, at 1,500 g for 15 minutes. After this last centrifugation, the PRP resulted divided in two different phases, collected as separate samples for the assays: 1) the supernatant containing K_3EDTA-PPP; 2) the platelet pellets. This centrifugation also allowed obtaining the heparinized plasma from the lithium heparin tube and the serum from the clot activator tube. All these different kinds of specimens were separately aliquoted in high quality, low binding protein Eppendorf Safe-Lock test tubes (Sigma-Aldrich, St. Louis, Mo, USA), while platelet pellets were maintained in the Falcon tubes. In particular, for each patient, two aliquots of platelet pellets and a total of around 16-18 aliquots for plasma and serum were obtained. The initial PRP volume, from which PPP and platelet pellets were derived, was registered and saved in the database for the calculation of intra-platelet 5-HT levels. Subjects' codes, dates and sample types were recorded on all the tubes, which

subsequently were frozen and stored at -80°C until the time of the assays (see **figure M2**).

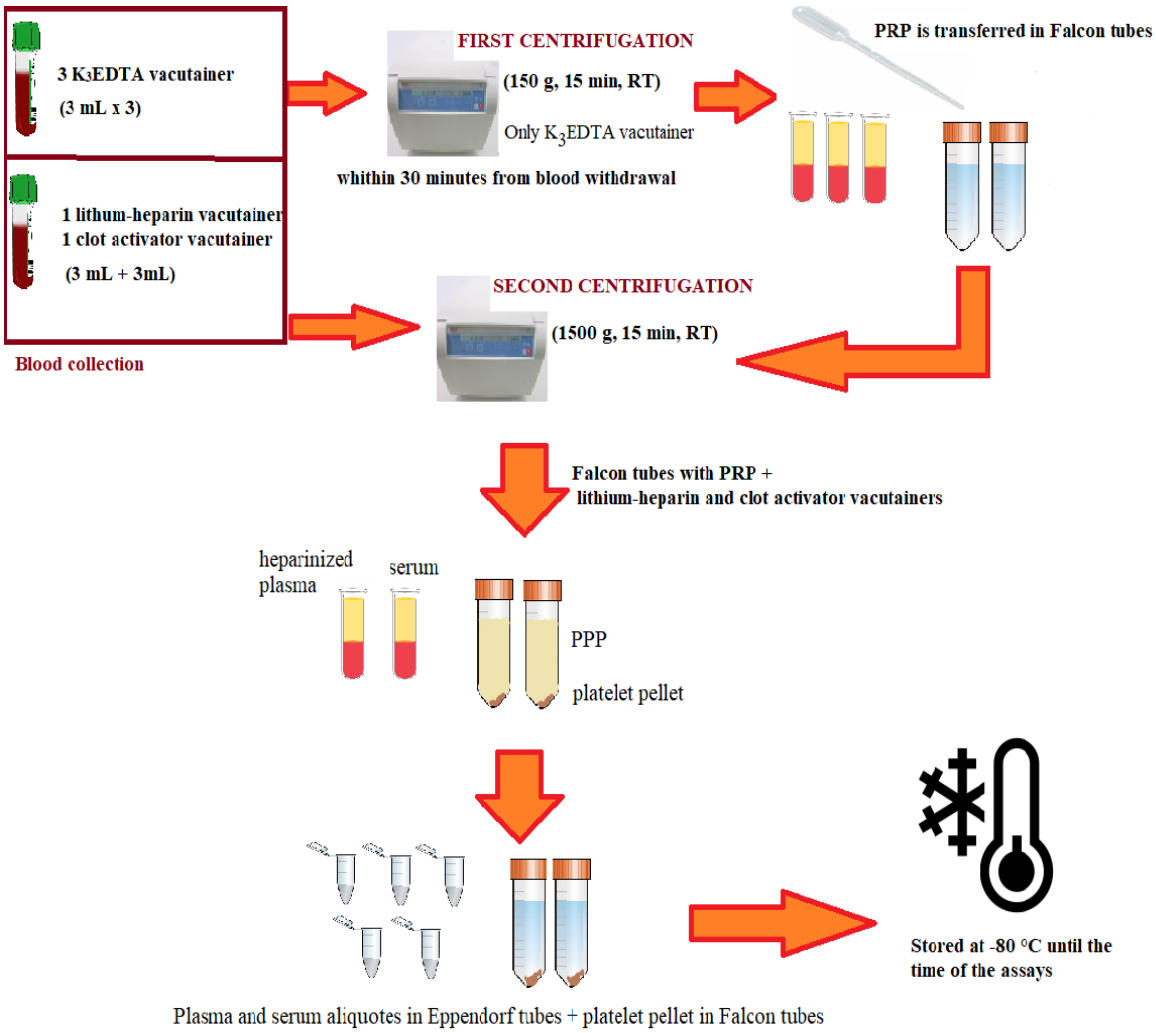

Figure M2. Blood sample preparation procedures.

3.3.3 ELISA tests for the determination of biochemical parameters

In order to evaluate the concentrations of the chosen biochemical parameters in our sample we used the Enzyme-linked Immunosorbent Assay (ELISA) technique, which is a specific and sensitive methodology featuring a double biological specificity, the specificity of the antibodies for antigens and the specificity of the enzyme catalytic site for its substrate. According to its name, the ELISA is an immunosorbent assay

associated with a revealing system featuring an antibody, or other binding-specific kinds of proteins, such as the streptavidin-biotin complex, conjugated to an enzyme.
Competitive, non-competitive and sandwich ELISA protocols are available. Each of them includes an immunological analysis which allows evaluating the presence and the amount of the analyte by the use of one or more antibodies, with one of them being directly or indirectly associated with an enzyme. This latter is the revelation device for the quantitative analysis and catalyzes a reaction, transforming a chromogenic/fluorogenic substrate into a colored/fluorescent/chemiluminescent product, which can be measured by a spectrophotometer or by a multimodal detection system. The ELISA is a highly versatile technique, allowing the application of several different approaches for different kinds of specimen and analytes, and thus offering a simple method to measure the chosen analytes in almost any kind of biological sample (serum, plasma, saliva, urine, tissue extract specimens or cell culture supernatants). Moreover, by means of ELISA methods, several samples can be measured simultaneously, in relatively short times. However, there are also some limitations linked to the use of this method. In particular, it can be affected by possible antibody cross-reactions and loss of the specificity for the analytes; moreover, the assay is unable to separate the analytes from the other sample components or to localize them within cells, potentially leading to a loss of analytical accuracy through the phenomenon known as “matrix effects” (Selby 1999). At the same time, due to the use of 96-well microtiter plates, the ELISA methodology remains a valuable alternative to the HPLC or the Ultra-HPLC (UHPLC) separation methodologies when there is the need to measure several different samples of the same analyte. In the present work, for the determination of the biochemical parameters, we used previously validated and commercially available ELISA kits: for measuring 5-HT, TRP, KYN, KYNA and QUIN levels we used indirect competitive ELISA kits produced by ImmuSmol (Bordeaux, France); for the evaluation of HCY levels an indirect competitive ELISA kit was employed, purchased from Alpha Diagnostic International (San Antonio, TX, USA); a sandwich ELISA kit produced by Biosensis (Thebarton, Australia) was instead used for quantifying BDNF levels, and for IL-6 a sandwich ELISA kit by Bio-Boster Biological Technology was employed (Pleasanton, CA, USA).

3.3.3.1 Assays for TRP, 5-HT and KYN pathway metabolites (KYN, QUIN, KYNA)

For the quantitative analysis of these parameters, a similar kind of ELISA procedure, developed by ImmuSmol® (Bordeaux, Francia), was used. The provided technical instructions for each kit were accurately followed during the analysis. TRP, 5-HT, KYN and QUIN were measured in PPP samples, KYNA was measured in serum. Intra-platelet 5-HT concentrations were also evaluated. The immune-enzymatic assays were developed for accurately measure low molecular-weight analytes through a derivatization step. Derivatization is a chemical reaction which modifies the analyzed compound in order to allow a better identification and quantification. The derivatization was performed in both standards and samples under investigation. In the ELISA kit used for TRP evaluation, the derivatization was conducted with a patented reagent, while for all the other metabolites a preliminary acylation reaction was performed. Each ELISA kit featured specific initial steps. For TRP and KYNA only, a preliminary extraction step was performed, following two distinct procedures: for TRP, samples were treated in Eppendorf tubes with an acidic solution in order to precipitate proteins (deproteinization step by a precipitating reagent), then removed by centrifugation; for KYNA, samples were treated by an acidic buffer and the extraction procedure was carried out in a dedicated extraction plate, then completed through a washing step using an extraction washing buffer provided by the kit. Once extracted, samples were derivatized prior to ELISA assay. For KYN, QUIN and 5-HT, a derivatization reaction through acylation was performed, without extraction/deproteinization. For intra-platelet 5-HT evaluation, platelets were defrosted until they reached RT, they were lysed in 1 mL of milliQ distilled water with 1 % of stabilizing agent (ascorbic acid) and then centrifuged at 10,000 g for 2 minutes at 25 °C. All the kits were provided with 6 ready standard solutions at known concentration for the quantitative measurement: for 5-HT, the concentrations ranged from 0 to 2.5 ng/ mL; for TRP, from 0 to 122 μM; for KYN, from 0 to 10,000 ng/mL; for QUIN, from 0 to 2031.77 ng/mL; for KYNA, from1.89 to 73.97 ng/mL. Only in the case of 5-HT the provided standard solutions must be preliminarily 1:1000 (*vol.:vol.*) diluted in assay buffer in order to obtain the required concentrations. All the kits provided two kinds of 96-well microtiter plates: one plate was dedicated to the analyte derivatization and the other for the actual ELISA assay.

The derivatization procedure had to be performed for all the kits, in both standards and samples, although, for each analyte, there was some variation, such as incubation times and the use of a thermo-shaker. After derivatization, a same volume of standards, samples and controls (the volume varied depending on the kit/analyte) was added to the second micro-plate wells in order to carry out the ELISA assay. The ELISA procedure featured several steps. The first step consisted in the competitive reaction and an overnight incubation. The assay micro-plate contained, pre-adhered in the bottom of each well (coating), the same derivatized analyte object of investigation. To realize the competitive reaction, the added derivatized analytes competed with the pre-adhered derivatized analyte for a specific antibody which was also added in equal volume to all the wells. The overnight incubation time for this reaction was about 15-20 hours, at 4°C. After incubation, the second step consisted in the washing procedures, in order to eliminate the unbound excess of reagents: 3 washes with 300 μL of washing buffer were carried out. Finally, the detection reaction was started, adding a secondary antibody linked to the horseradish peroxidase (HRP) enzyme. Through this procedure, which is of the indirectly competitive type, the second antibody associated with the enzyme is bound depending on the quantity of the first antibody which previously adhered on the bottom of the well (see **figure M3**). After further washing steps, the 3,3′,5,5′-Tetramethylbenzidine (TMB), a HRP substrate, was added to the wells avoiding light exposure: the incubation time for this enzymatic reaction was around 30 minutes at 25 °C in the thermo-shaker (600 rpm). After the incubation, the reaction was stopped through precipitation and inactivation of the HRP by adding a stop solution containing concentrated sulfuric acid (H_2SO_4). Due to the acidic pH, the blue colored product of the enzymatic reaction changes color, developing a yellow solution. Since these methods are all competitive, the intensity of the color decreases at the increasing of the analyte concentration. The final absorbance in each micro-well was measured by the plate reader spectrophotometer at $\lambda = 450$ nm within 10 minutes from the adding of the stop solution. The standard curve for the quantitative analysis of each analyte was then calculated by evaluating the absorbance values of the standards and performing a non-linear regression at 4 logistic parameters (4-parameter logistic regression). The regression had a negative slope value due to the inverse proportion between the analyte concentration and the color intensity. The final concentrations of the analytes in the

samples were then interpolated from the calibration curve, considering the dilution factor when necessary.

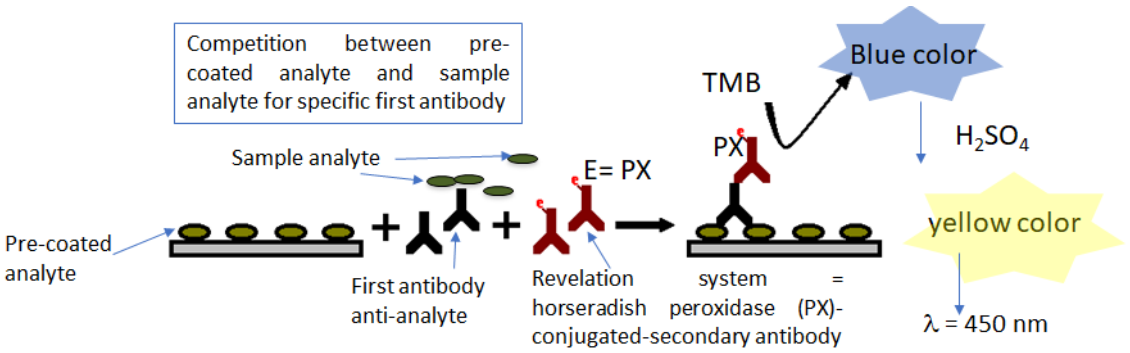

Figure M3. Schematization of indirect competitive ELISA.
TMB = Tetramethylbenzidine.

3.3.3.2 Assays for HCY levels

The principle of the assay kit employed herein to measure HCY levels in plasma was based upon an indirect competitive ELISA procedure, similar to that used to determine TRP metabolites. In this case, prior to the ELISA dosage, plasma samples were incubated with an enzyme reaction mixture, containing the enzyme S-adenosyl-L-homocysteine (SAH) hydrolase and its substrate adenosine/dithiothreitol (DTT), in order to transform the whole amount of HCY into SAH. This passage is required in order to avoid matrix effects that may occur when a direct measure of HCY is performed. The HCY transformation into SAH was carried out in a thermostatic bath, at 37°C, and stopped by the addition of a specific enzyme inhibitor at 18-25°C, followed by a further stopping step in the presence of the enzyme adenosine deaminase, to deactivate adenosine, the substrate of the SAH formation reaction. At this point, the ELISA procedure was performed. The ELISA microplate assay of the kit was pre-coated with SAH. The calibration curve ranged from 2 to 50 µM. The competition was carried out by means of an incubation step with a monoclonal anti-SAH mouse antibody, followed by a subsequent stage in the presence of a secondary biotinylated

anti-mouse antibody. As for the other assays, the revelation step consisted in the addition of a biotin-streptavidin complex coupled to HRP and then TMB.

3.3.3.3 Assays for BDNF levels

For BDNF dosages, a sandwich ELISA kit provided by Biosensis, developed for the preferential determination of the BDNF mature form (Biosensis, mature BDNF RapidTM, Thebarton, Australia) was used. This kit allowed the colorimetric identification and quantification of BDNF levels in different kinds of biological specimens. It is validated to prevent analytical interferences and its quantitative performances are certified. The antibodies used in the kit react only with the mature form of BDNF, avoiding cross-reactions with the precursor pro-BDNF. The test has a high sensitivity, allowing the detection of BDNF concentration as low as 7 pg/mL, and a high specificity, with less than 3 % of other neurotrophins (such as NT-3, NT-4 and NGF) that can be bound by the first monoclonal anti-BDNF "capture" antibody. The kit thus includes this first monoclonal antibody pre-coated on the bottom of the 96-well micro-plate, a second biotinylated detection antibody directed towards another BDNF epitope, and a streptavidin-biotin detection complex conjugated with HRP. By adding the TMB substrate, a blue reaction product is generated, proportional to the BDNF concentrations in standards and samples. The kit also provides a validated human recombinant lyophilized BDNF standard at a certified concentration, together with a quality control sample which functions as a positive control of BDNF at a validated concentration range. Before the test, the lyophilized BDNF standard must be diluted in 1mL of sample diluent buffer in order to reach a final concentration of 1 ng/mL (1000 pg/mL). This first solution is then serially diluted in the sample diluent buffer in order to obtain 7 calibration solutions, which will have a BDNF concentration ranging from 7.8 to 500 pg/mL. The sample diluent buffer includes some blocking components, which allow preventing the formation, on the bottom of the micro-plate wells, of non-specific bindings and the consequent possible high background values (noise) at the end of the test. The day of the assay, the aliquots of defrosted PPP of subjects were diluted in the sample diluent buffer as requested by the kit guidelines. Subsequently, mature BDNF diluted standard, the quality control sample, the diluted PPP samples from the subjects and "the blanks" (containing only the sample diluent buffer) were added to the wells of the micro-plate. The plate was then covered with an appropriate plate sealer

film for the incubation on a thermo-shaker at very low shaking speed. After the incubation, the procedure featured 5 washing steps. Then, diluted biotinylated anti-mature BDNF antibody was added in each well, and the plate underwent a further incubation on the thermo-shaker. Subsequently, after a further washing phase, diluted streptadivin-HRP complex was added to all the wells and then the plate was incubated again at very low shaking speed. To reveal the immunocomplex formation, TMB, the HRP substrate, was added in each well: this reaction was stopped after around 6-7 minutes by adding the stop solution. As reported for the other dosages, this latter step changes the blue color of the HRP reaction product into yellow, yielding, in this case, a color intensity directly proportional to BDNF concentrations (see also **figure M4**). Micro-well absorbance was then measured by the plate reader spectrophotometer, presetted at λ = 450 nm. For calculating BDNF values, the blank absorbance (Abs_{450} BDNF= 0 pg/mL) was subtracted from absorbance of both standards and samples. The BDNF values were then interpolated from the calibration curve and multiplied by dilution factor in order to obtain the final levels of BDNF in the PPP as ng/mL.

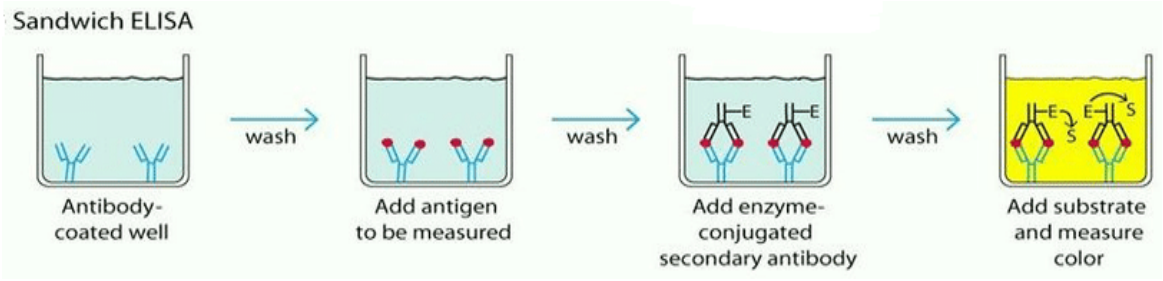

Figure M4. Schematization of sandwich ELISA (from Alahi and Mukhopadhyay 2017).

3.3.3.4 Assays for IL-6 levels

IL-6 concentrations were measured in PPP specimens by means of a sandwich ELISA kit (Picokine IL-6 assay, Boster Biological Technology, Pleasanton, CA, USA), following a procedure very similar to the BDNF assay one. Before performing the assay, aliquots of defrosted PPP samples were diluted in the sample diluent buffer, according to the kit guidelines. In particular, the kit utilizes a first monoclonal antibody,

in this case an anti-IL-6 antibody, then a second biotinylated antibody, as well as a complex of streptavidin-biotin-peroxidase as the signal-to-noise ratio amplifier. The standard calibration range used to calculate the calibration curve was 4.69-300 pg/mL. Plate absorbance was spectrophotometrically read at λ = 450 nm. The calibration curve was built by a 4-parameter logistic regression equation, to interpolate IL-6 concentrations in unknowns as pg/mL. This method features a high sensitivity, with a determination limit of 0.3 pg/mL.

3.3.3.5 Determination of total proteins according to the Bradford's method

This procedure was used for normalizing the intra-platelet 5-HT concentrations for the quantity of total proteins in each platelet soluble fraction. This method presumes that the protein content in each sample would be proportional to the number of the platelets separated by the PRP centrifugation and to the final total protein recovery after platelet lysis and lysate centrifugation. Normalizing the analyte intra-platelet concentration for the total protein allows avoiding altered values associated with the high intra- and inter-individual variability of platelet counts. It also allows considering possible different yields of platelets and proteins. The colorimetric method of Bradford is a simple procedure of fast execution endowed with a high sensitivity (Bradford 1976). It can be used also when protein concentration is very low (< 25 μg/mL), and is considered advantageous for rapid determinations in 96-well micro-plates. The procedure can be performed by a unique step: protein standards and samples were diluted in distilled water (20:80 v/v) and then Bradford dye (Coomassie Brilliant Blue G-250) was added to all wells. In acidic conditions, the Bradford dye forms complexes with aromatic and basic amino acids contained in sample proteins. The measure is realized by changing the dye λ_{max} absorbance from 465 to 595 nm, which generates a blue color whose intensity is proportional to the total protein content. The hydrophobic aromatic amino acids and the positively charged basic amino acids of the proteins bind the Bradford dye through electrostatic and hydrophobic non-covalent reactions. The spectrophotometer can read the plate at 595nm: due to the high stability of the complex, it is possible to perform the analysis within 1 hour from the moment of the addition of the Bradford dye. The day of the assay, in order to obtain the concentrations for the standard curve, a γ-globulin

solution (0.1 mg/mL) was diluted in milliQ water. Platelet soluble fractions were also properly diluted in milliQ water. After the addition of the Bradford dye to samples and standards, tubes were gently shaken and immediately read by the spectrophotometer at 595 nm. The protein content was interpolated from the calibration line. Values of intra-platelet 5-HT (ng/mL) were normalized for the protein content (mg/mL) and reported as ng/mg protein (see **figure M5**).

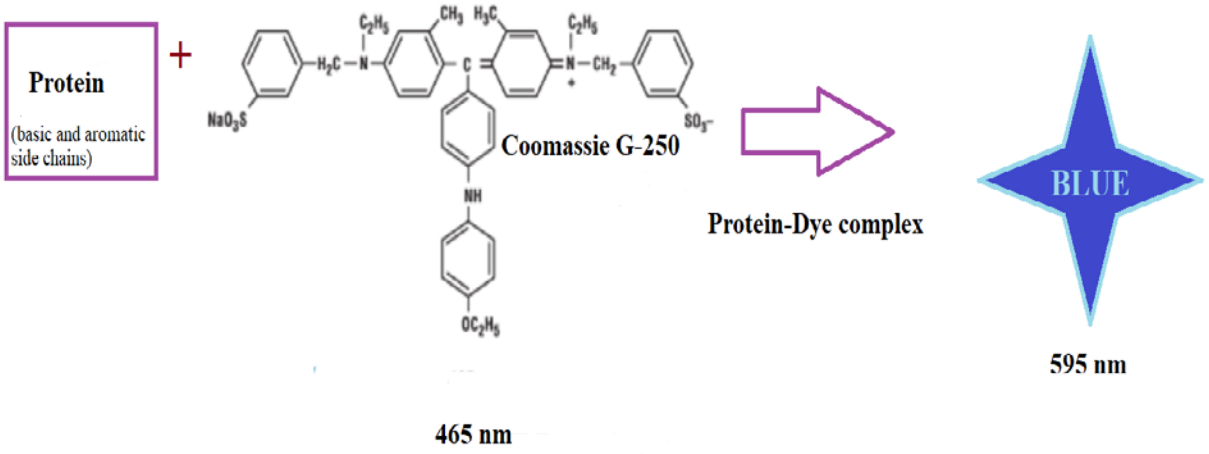

Figure M5. Schematization of Bradford's method for determination of total proteins (Bradford 1976).

3.4 Statistical analysis

For the biological parameters investigated here, the final concentrations in plasma or serum were interpolated as follows: all concentration units of measure were transformed in log values, $x = \log_{10}(\text{conc})$, and a semi-log calibration curve (y vs. logx) was built by means of a 4-parameters logistic non-linear regression. After interpolating concentration results in unknowns, log values were then retransformed into the non-logarithmic measures ($x = 10^x$).

Considering that normality tests and variance homoscedasticity were not respected in our sample, we used non-parametric analyses for elaborating our data. A Kruskal-Wallis one way analysis of variance was used in order to compare continuous socio-demographic variables, psychometric instrument scores and biochemical parameter concentrations among groups, followed by Dunn test for *post-hoc* comparisons. For comparing biochemical parameter concentrations depending on the presence of specific diagnoses or pharmacological treatments, the Mann-Whitney U-test was used. Chi-square tests were performed in order to compare categorical socio-demographic variables among groups. We performed a Spearman's correlation coefficient (r) in order to evaluate the correlations between different biochemical variables and between biochemical variables and the psychometric instrument scores. We also performed a multinomial logistic regression in order to identify which biochemical parameters were statistically predictive of being included in the ASD or in the BAP group. All the analyses were performed using SPSS version 24 (IBM Corp. 2016) and GraphPad Prism (Versione 7.0, San Diego, USA). GraphPad was used also for calculating the calibration curves and the regression analysis for each biochemical assay. For all the statistical analyses performed in this study, the statistical threshold was set at $p \leq .05$.

4. Results and discussion

4.1 Socio-demographic features and psychometric scales

Results

Our sample was composed of 24 subjects with ASD (ASD group), 24 unaffected relatives of ASD patients (BAP group) and 24 unrelated healthy controls (CTL group). The mean age was 27.75±6.97 years for the ASD group, 55.42±10.25 years for the BAP group and 33.29±8.05 years for the CTL group. Subjects in the BAP group were significantly older than those in the other two groups. Regarding sex composition, the ASD group included 17 M and 7 F, the BAP group 3 M and 21 F and the CTL group 9 M and 15 F. The ASD group was composed in a significantly higher proportion by males (70.8 %) when compared with the other two groups, with the CTL group also reporting a significantly higher proportion of males with respect to the BAP group (37.5 % vs. 12.5 %). Details are reported in **Table 1**.

		ASD (n=24) *(Mean±SD, Mean rank)*	**BAP (n=24) *(Mean±SD, Mean rank)***	**CTL (n=24) *(Mean±SD, Mean rank)***	**H**	**p***
Age, years		27.75±6.97, 20.77	55.42±10.25, 57.71	33.29±8.05, 31.02	39.92	**<.001**[*]
Mean BMI, Kg/m²		26.32±7.14, 41.69	25.34±6.32, 37.31	23.02±3.92, 30.50	3.484	.175
					Chi-square	**p**
Sex n(%)	**M**	17(70.8%)	3(12.5%)	9(37.5%)	17.09	**<.001**[#]
	F	7(29.2%)	21(87.5%)	15(62.5%)		

Table 1. Socio-demographic features of the sample.

Significant post-hoc comparisons:

[*] *BAP>CTL>ASD, p<.05*

[#] *M: ASD>CTL>BAP, p<.05*

Almost all of ASD subjects reported other current diagnosis in comorbidity (91.67 %, n = 22). In particular, the most frequently reported diagnosis was Bipolar I disorder (41.67 %, n = 10), followed by Bipolar II disorder (29.17 %, n = 7) and anxiety

disorders (29.17 %, n = 7), few subjects were affected by Obsessive-compulsive disorder (12.50 %, n = 3), Feeding and eating disorders (12.50 %, n = 3), and Post-traumatic stress disorder (PTSD) (4.16 %, n = 1). Also in the BAP group, several subjects (45.83 %, n = 11) were currently affected by at least one psychiatric disorder. In particular, the more frequent disorders were anxiety disorders (37.50 %, n = 9), followed by Major depressive disorder (12.50 %, n = 3), while only 1 subject (4.16 %) was affected by Bipolar II disorder and another one (4.16 %) by Feeding and eating disorders. Considering that the sample was composed of adult subjects followed at a psychiatric department as in- or out-patients, almost all the members of the ASD group (n = 23) were under pharmacological therapy. In particular, 10 subjects (41.67 %) were taking antidepressants, 12 (50 %) anxiolytics, 12 (50 %) lithium, 18 (75 %) were taking other mood stabilizers (antieplieptics) and 22 (91.67 %) antipsychotic drugs. All the treated subjects were taking more than one kind of drug. In the BAP group, a total of 6 subjects (25 %) were under pharmacological therapy: 4 subjects (16.67 %) were taking antidepressants, while 2 (8.33 %) were taking anxiolytics and 2 (8.33 %) were taking antiepileptic drugs. Details about socio-demographic features, psychiatric comorbidity and pharmacological treatments of the sample are reported in **Table 2**.

We also performed a comparison of the scores reported at the psychometric scales among groups. For each instrument, higher scores are indicative of higher autistic traits and/or a higher impairment of the specific function investigated. For all the scales measuring autism spectrum symptoms and traits (AdAS Spectrum, AQ, RAADS-14), ASD patients reported significantly higher total scores than the other groups. However, also the BAP group reported significantly higher total scores than the CTL group. Similar results were reported for the single domains of the instruments: considering the AdAS Spectrum, ASD patients reported significantly higher scores than BAP and CTL groups on all domains with the exception of *Empathy*, for which BAP and ASD subjects did not significantly differ. BAP group reported, in turn, significantly higher scores than CTL group on all domains but *Childhood/adolescence,* for which no significant difference was found between BAP and CTL groups. ASD subjects reported significantly higher scores than the other two groups also on all AQ and RAADS-14 domains, with the exception of the AQ *Social skill* and *Imagination* domains, for which the scores reported by ASD and BAP subjects were not significantly different.

	ASD (n= 24) *n (%)*	**BAP (n=24)** *n (%)*	**CTL (n=24)** *n (%)*
Psychiatric disorders			
Anxiety disorders	7(29.17%)	9(37.50%)	-
Obsessive-compulsive disorder	3(12.50%)	0(0%)	-
Major depressive disorder	0(0%)	3(12.50%)	-
Bipolar I disorder	10(41.67%)	0(0%)	-
Bipolar II disorder	7(29.17%)	1(4.16%)	-
Feeding and eating disorders	3(12.50%)	1(4.16%)	-
PTSD	1(4.16%)	0(0%)	-
At least one psychiatric disorder (other than ASD)	22(91.67%)	11(45.83%)	
Pharmacological treatment			
Antidepressants	10(41.67%)	4(16.67%)	-
Anxiolytics	12(50%)	2(8.33%)	-
Lithium	12(50%)	0(0%)	-
Other mood stabilizers (Antiepileptics)	18(75%)	2(8.33%)	-
Antipsychotics	22(91.67%)	0(0%)	-
At least one pharmacological treatment	23(95.83%)	6(25%)	

Table 2. Psychiatric disorders and pharmacological treatments among groups.

Moreover, BAP subjects reported significantly higher scores than CTL subjects on most of the AQ and RAADS-14 sub-scales, although not reporting significantly different scores with respect to the CTL group on AQ *Attention switching, Attention to detail* and

Imagination domain scores and RAADS-14 *Social anxiety* domain scores. On the RRS, the scale which specifically measures the autism-related dimension of ruminative-thinking, ASD subjects reported significantly higher scores than CTL and BAP groups on the total and on all domains scores, with the BAP group showing significantly higher scores than the CTL group. Finally, similar results were also reported for the WSAS, which measures social and work adjustment: ASD patients scored significantly higher (lower functioning) on all WSAS items and on the total than the CTL group, reporting also significantly higher scores than the BAP group for all the items but the one investigating the adjustment/functioning in *Close relationships,* for which no significant differences were found in the scores reported by ASD and BAP subjects. BAP group also reported significantly higher scores than CTL group on all the items and the total, with the exception of the items which investigate the subject's functioning in *Work* and *Home management* areas. Results are reported in **Table 3.**

	ASD (n=24) ***(Mean±SD, Mean rank)***	**BAP (n= 24)** ***(Mean±SD, Mean rank)***	**CTL (n=24)** ***(Mean±SD, Mean rank)***	**H**	**p**
AdAS Spectrum					
Childhood/adolescence	10.46±3.69, 51.52	5.57±3.37, 31.33	3.26±2.14, 19.63	31.58	**<.001**+
Verbal communication	10.13±4.88, 53.77	4.19±2.34, 32.45	1.57±1.67, 16.26	42.95	**<.001***
Non-verbal communication	12.50±4.41, 52.53	6.95±3.60, 32.88	3.57±2.25, 17.48	36.67	**<.001***
Empathy	5.25±2.79, 49.29	3.14±2.48, 36.55	0.82±1.03, 17.20	31.99	**<.001**#
Inflexibility and adherence to routine	21.25±6.77, 52.60	12.24±5.97, 34.67	5.39±3.46, 15.46	41.59	**<.001***
Restricted interests and rumination	12.75±4.30, 51.90	7.24±4.12, 34.69	3.09±1.90, 16.17	38.54	**<.001***
Hyper-hypo reactivity to sensory input	6.46±3.44, 50.81	3.10±2.34, 34.50	0.87±1.22, 17.48	34.11	**<.001***
Total score	78.79±21.94, 55.04	42.43±16.85, 33.79	20.83±13.41, 16.02	45.54	**<.001***
AQ					
Social skill	5.38±2.22, 40.33	3.88±1.83, 32.12	1.00±1.91, 13.68	27.13	**<.001**#
Attention switching	6.86±1.96, 40.67	4.18±1.85, 23.21	3.61±2.09, 19.31	19.50	**<.001**+

Attention to detail	5.95±2.38, 39.12	3.24±1.68, 21.53	3.44±1.98, 22.69	14.55	**<.001**[+]
Communication	5.33±1.98, 42.36	3.35±1.84, 29.82	1.26±0.93, 13.50	30.77	**<.001**[*]
Imagination	5.05±2.25, 39.10	3.47±1.87, 27.79	2.42±1.30, 18.92	15.15	**.001**[°]
Total score	30.16±4.99, 42.34	19.24±4.13, 24.91	11.31±3.38, 9.38	41.47	**<.001**[*]
RAADS-14					
Mentalizing deficits	11.43±4.85, 51.86	4.00±5.00, 31.25	0.36±0.95, 16.75	40.26	**<.001**[*]
Social Anxiety	6.81±3.08, 51.40	2.23±2.69, 30.39	0.09±0.29, 18.05	38.76	**<.001**[+]
Sensory reactivity	10.46±3.69, 48.05	4.45±3.13, 32.95	3.45±2.63, 18.68	28.97	**<.001**[*]
Total score	22.81±8.36, 52.36	8.27±8.53, 32.70	0.77±1.44, 14.82	43.67	**<.001**[*]
RRS					
Reflection	12.09±3.06, 47.29	8.90±2.61, 32.36	6.41±1.94, 16.36	30.6	**<.001**[*]
Brooding	13.14±3.04, 48.93	9.68±2.71, 33.32	6.71±1.49, 15.21	34.81	**<.001**[*]
Depression	32.71±6.65, 49.57	23.19±4.62, 31.24	17.62±3.84, 15.19	37.09	**<.001**[*]
Total score	57.95±10.74, 49.38	41.05±8.86, 30.20	30.76±6.53, 14.86	38.66	**<.001**[*]
WSAS					
Work	5.25±2.02, 50.35	2.00±2.29, 30.23	0.41±0.73, 18.55	33.56	**<.001**[+]
Home management	4.70±2.76, 47.60	1.82±2.15, 31.36	0.32±0.65, 19.91	26.11	**<.001**[+]
Social leisure activities	4.75±2.63, 47.50	2.27±2.64, 33.52	0.23±0.53, 17.84	29.29	**<.001**[*]
Private leisure activities	5.40±2.66, 47.80	2.95±2.98, 34.34	0.14±0.35, 16.75	32.32	**<.001**[*]
Close relationships	3.65±3.03, 44.15	1.91±2.02, 34.59	0.09±0.29, 19.82	21.94	**<.001**[#]
Total score	23.75±9.77, 49.98	10.95±10.20, 33.02	1.18±2.20, 16.09	36.22	**<.001**[*]

Table 3. Comparison of psychometric instrument scores among groups.

Significant post-hoc comparisons:

[*] *ASD>BAP>CTL, p<.05*

[+] *ASD>BAP, CTL, p<.05*

[#] *ASD, BAP>CTL, p<.05*

[°] *ASD>CTL, p<.05*

Discussion

Significant age and sex differences were reported among groups. The age difference was linked to the fact that most of the BAP subjects were parents of ASD patients (only in two cases the relative who chose to participate, or who was eligible for participation, was a sibling). Moreover, in most of the cases, the involved parent was the mother, because the father was not available or refused to participate. This data, while accounting for the higher prevalence of females in the BAP group, may be in line with previous research in this field, which stressed the lack of father involvement in several aspects of parental care, with a greater burden on mothers as caregivers (Johnson and Simpson 2013). On the other hand, the higher proportion of males in the ASD group should be considered in light of the greatly higher prevalence of ASD in the male gender (APA 2013) (see also **chapter 5**, Limitations and conclusion).

The high presence of comorbid disorders in ASD patients is in accordance with previous literature, which underlined not only the high prevalence of psychiatric comorbidities, and in particular mood and anxiety disorders, in ASD, but also how ASD it-self would be a risk-factor for developing other psychiatric disorders (Ghaziuddin and Zafar 2008; Dell'Osso et al. 2016; 2019a). Shared genetic underpinnings were also stressed among ASD, Bipolar disorders and Schizophrenia (Carrol and Owen 2009: Dell'Osso et al. 2019b). Moreover, we recruited in this study adults with milder forms of ASD (without intellectual impairment or language development alteration) who were medically followed at a psychiatric clinic: this particular population was reported to often come to clinical attention and seek help only after the development of other disorders in comorbidity, due to an under-recognition of ASD symptoms during childhood or early adolescence (Dell'Osso et al. 2016; 2019a). Given this context, it could be possible that in our ASD population other psychiatric disorders were particularly highly represented. As a consequence, most of the patients were also under pharmacological treatment. The particularly high percentage of patients under treatment with antipsychotics can be accounted to the fact that, while there are no specific drugs approved for treating ASD core symptoms, the only two drugs approved for the treatment of specific symptoms associated with ASD, such as irritability (while not considering other comorbidities), lie in the class of antipsychotics (risperidone and aripiprazole) (Denucci et al. 2021). Psychiatric disorders, and in particular anxiety and mood disorders, were represented also in the BAP group, with a higher prevalence than

in the general population (Faravelli et al. 2004; de Girolamo et al. 2006). This result is in line with previous literature, which stressed the presence of higher psychiatric symptoms and full-fledged disorders among relatives of ASD patients with respect to the general population and also to parents of children with neurological disorders such as Down's syndrome (Piven et al. 1991; Carpita et al. 2020b). Further authors pointed out that psychiatric conditions in this population would often onset before the birth of the child, thus excluding that the stressful, eventually traumatic consequences of being a caregiver of an ASD child would be accountable for the increased presence of anxiety/mood disorders among the parents (Ingersoll and Hambrick 2011; Carpita et al. 2020b). This data may also confirm the reported familial aggregation between ASD and other psychiatric disorders (Sullivan et al. 2012). Moreover, it was reported that, among relatives of ASD probands, having a psychiatric disorder would be linked to (and statistically predicted by) the presence of more severe autistic traits, leading to further stress the possible role of autistic traits as vulnerability factors towards psychopathology, also when sub-threshold, as well as the phenotypic continuity and the shared genetic underpinnings between ASD and BAP (Carpita et al. 2020b) (see also **chapter 5**, Limitations and conclusion).

The intermediate scores reported by the BAP group on all the autism-related psychometric scales, showing a significant difference with respect to the other two groups (lower than ASD but higher than CTL) for total scores and most of the instruments' domains, are in line with previous literature. In particular, these results confirmed the theoretical framework of our study, stressing the continuity of autistic traits between ASD patients and their relatives (which was the main basis of the conceptualization of BAP) and the possible identification of the group of relatives, also in our sample, as subjects showing an intermediate expression of the autism spectrum from a psychopathological point of view (Folstein and Rutter 1977; Bailey et al. 1988; Losh et al. 2008; 2009; Wheelwright et al. 2010; Billeci et al. 2016; Carpita et al. 2020b). Considering specific dimensions and the impact on functioning, our results seem to suggest, in BAP, a particular higher impairment of social skills and empathy (although with lower associated levels of social anxiety with respect to the ASD group). A worse functioning in leisure and relational areas due to the reported symptoms was alo observed: noticeably, the scores reported by ASD and BAP group for the relational area did not significantly differ and were instead significantly higher than those of the

CTL group. BAP group seems to instead maintain a better adjustment in work and home management, and a lower impairment of attention mechanisms. These findings globally confirm those from previous studies, which highlighted that the impairment of social and relational skills seems to be the most marked autistic-like feature in parents of ASD patients (Gerdts and Bernier 2011). Finally, the higher functional impairment of BAP with respect to the CTL group (although still lower than that reported in ASD patients) also confirms results from previous studies which reported the presence of lower adjustment in this population, further highlighting the actual impact of autistic traits, also when sub-threshold, on life quality (Carpita et al. 2021b).

4.2. Comparison of biochemical parameters among groups

Results

Results from the comparison of the levels of biochemical parameters among groups showed that PPP 5-HT levels were significantly lower in the ASD group with respect to the other two groups while intra-platelet 5-HT levels were significantly lower in the ASD group only when compared with controls.

For TRP and the metabolites of the KYN pathways, significant differences were found only for TRP and KYNA levels, while no difference was found in KYN and QUIN levels among groups. In particular, KYNA levels were significantly lower in the ASD group than in the other two groups. TRP levels were significantly lower in both ASD and BAP groups when compared with CTL subjects. In order to better evaluate the TRP metabolism, we also compared the ratio between parameters among groups. KYNA/KYN ratio was significantly lower in the ASD group when compared with CTL subjects. Moreover, KYNA/TRP and KYNA/QA ratios were significantly reduced in the ASD group only when compared with the BAP group. No significant difference was found for 5-HT (PPP)/TRP, QA/KYN, QA/TRP and KYN/TRP ratios.

Both IL-6 and HCY levels were found significantly higher in the ASD group only when compared with CTL subjects, with BAP scoring intermediately between the other two groups. We did not find any difference also for BDNF levels (see **Table 4, Figure 1R** and **Figure 2R**). Reference values for HCY, whose concentrations are measured in

common laboratory screening procedures, ranges from 5-15 µM (hyper-HCY is defined by values above 15µM), although optimal values should be lower than 10 µM (Rehman et al. 2020). In our sample, 29.17 % (n = 7) of ASD group, 8.33 % (n = 2) of BAP group and no subject in the CTL group reported values above 15µM.

	ASD (n=24) *(Mean±SD, Mean rank)*	**BAP (n=24)** *(Mean±SD, Mean rank)*	**CTL (n=24)** *(Mean±SD, Mean rank)*	**H**	**p**
5-HT (PPP), ng/mL	19.998±41.151, 23.70	22.996±16.069, 41.36	27.085±38.792, 40.00	11.01	**.004**$^{+}$
5-HT (platelet), ng/mg proteins	10.087±17.514, 25.70	15.284±14.781, 39.27	12.805±7.553, 40.00	7.44	**.024***
TRP, µM	49.778±20.957, 29.30	49.895±17.566, 29.68	67.170±25.698, 45.33	9.77	**.008**$^{\#}$
KYN, ng/mL	535.729±275.166, 32.70	653.960±347.114, 38.18	563.579±304.097, 34.29	0.89	.642
KYNA, ng/mL	10.395±6.031, 23.28	16.525±7.486, 40.05	17.099±8.129, 41.60	11.84	**.003**$^{+}$
QUIN, ng/mL	49.429±30.435, 33.74	51.154±40.542, 32.55	53.282±26.70,4 38.46	1.13	.567
BDNF, ng/mL	5.779±6.436, 34.39	5.693±5.621, 37.14	4.932±5.491, 33.63	3.83	.896
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**$^{\circ}$
HCY, µM	12.939±8.485, 40.18	10.001±3.520, 36.95	7.507±2.339, 23.96	9.03	**.011**$^{\circ}$
5-HT (PPP)/TRP	0.002±0.005, 28.57	0.003±0.002, 42.61	0.002±0.003, 34.19	5.58	.061
QA/KYN	0.101±0.058, 35.72	0.085±0.041, 30.55	0.11±0.71, 38.40	1.80	.406
QA/TRP	0.006±0.004, 37.11	0.006±0.004, 36.93	0.005±0.004, 31.21	1.32	.518
KYN/TRP	0.057±0.026, 37.83	0.079±0.059, 39.52	0.051±0.046, 28.15	4.38	.112
KYNA/TRP	0.001±0.001, 27.85	0.002±0.001, 43.68	0.002±0.001, 33.90	7.15	**.028**§
KYNA/KYN	0.023±0.017, 25.76	0.035±0.026, 36.73	0.033±0.013, 42.27	8.19	**.017***
KYNA/QA	0.264±0.164, 26.43	0.436±0.289, 40.39	0.371±0.186, 38.27	6.42	**.040**§

Table 4. Comparison of biochemical parameters among groups.

Significant post-hoc comparisons:

**ASD<CTL, p<.05*

$^{+}$*ASD<BAP, CTL, p<.05*

$^{\#}$*ASD, BAP<CTL, p<.05*

$^{\circ}$*ASD>CTL, p<.05*

§*ASD<BAP, p<.01*

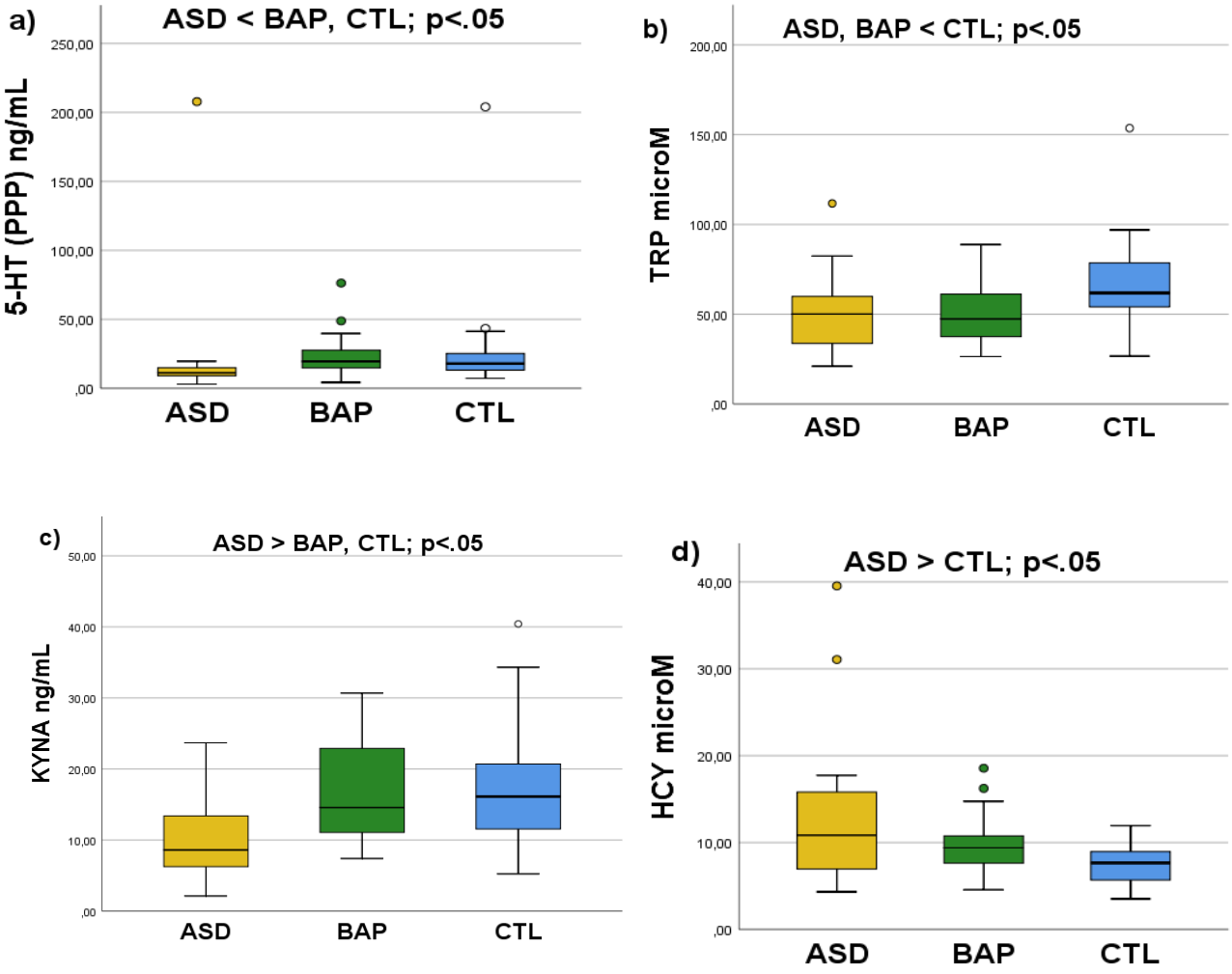

Figure 1R. Comparison of biochemical parameters showing significant differences among groups. 1Ra) 5-HT (PPP); 1Rb) TRP; 1Rc) KYNA; 1Rd) HCY; 1Re) IL-6 (see figure 3R for intra-platelet 5-HT).

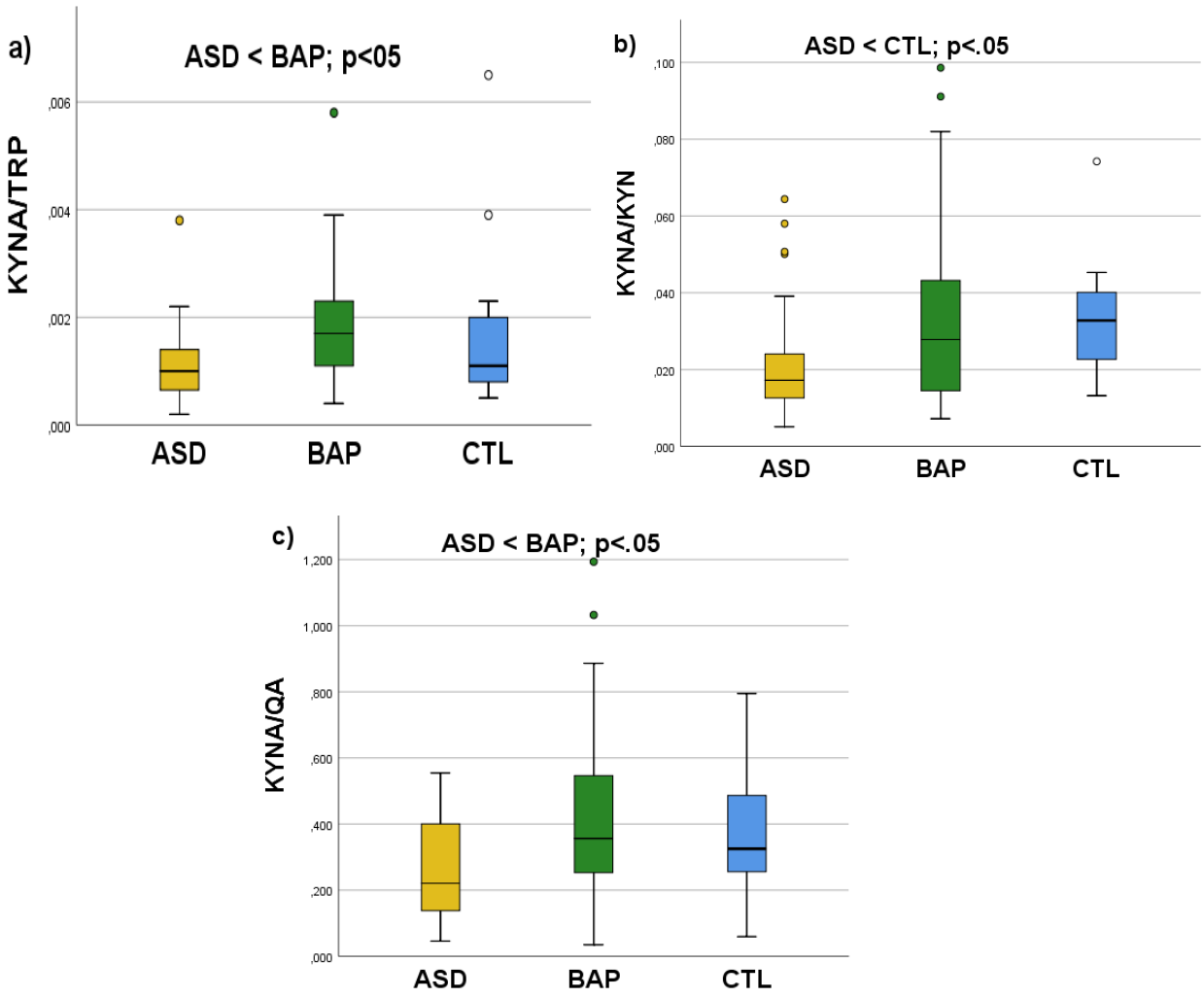

Figure 2R. Comparison of ratio between biochemical parameters showing significant differences among groups. 2Ra) KYNA/TRP; 2Rb) KYNA/KYN; 2Rc) KYNA/QUIN.

Subsequently, we evaluated the possible differences in the levels of biochemical parameters depending on the presence of specific pharmacological therapy and on the presence of the most frequently reported comorbid disorders, within the ASD and the BAP group. Regarding psychiatric comorbidity, we compared the levels of biochemical parameters depending on the presence of Bipolar I, Bipolar II or anxiety disorders in the ASD group and depending on the presence of Major depressive disorder or anxiety

disorders in the BAP group, as the most frequent diagnoses for each group, without finding any significant difference with respect to the levels of the analytes under investigation.

In the ASD group, no difference in biochemical parameters was found between subjects who were taking anxiolytic, antipsychotic or mood stabilizers (lithium or antiepileptics). On the other hand, we found that ASD subjects under treatment with antidepressants (n = 10) showed significantly lower levels of intra-platelet 5-HT than other ASD subjects (n = 14) (No antidepressants: mean = 16.962±21.054 ng/mg proteins, mean rank = 17.00; Antidepressants: mean = 1.150±1.311 ng/mg proteins, mean rank = 5.50; U = 0.00; $p < .001$). A similar result was found in the BAP group: significantly lower levels of intra-platelet 5-HT were found in subjects who were taking antidepressants (n = 4), with respect to the others (n = 20) (No antidepressants: mean = 18.297±14.719 ng/mg proteins, mean rank = 13.28; Antidepressants: mean = 1.731±1.558 ng/mg proteins, mean rank = 3.50; U = 4.00; $p = .003$); no difference was found for any biochemical parameter depending on the assumption of other drugs. On the basis of this data, we performed a further comparison of intra-platelet 5-HT levels among groups, excluding subjects in treatment with antidepressants: in this case, no significant difference was found: ASD and BAP group reported higher, although not significant, mean values than CTL subjects. Results are shown in **Table 5** and **Figure 3R.**

	ASD (n=14) ***(Mean±SD, Mean rank)***	**BAP (n=20)** ***(Mean±SD, Mean rank)***	**CTL (n=24)** ***(Mean±SD, Mean rank)***	**H**	**p**
5-HT (platelet), ng/mg proteins	16.962±21.054, 25.23	18.296±14.719, 32.33	12.805±14.048, 26.25	1.992	.369

Table 5. Comparison of intra-platelet 5-HT among groups after excluding subjects under treatment with antidepressant drugs.

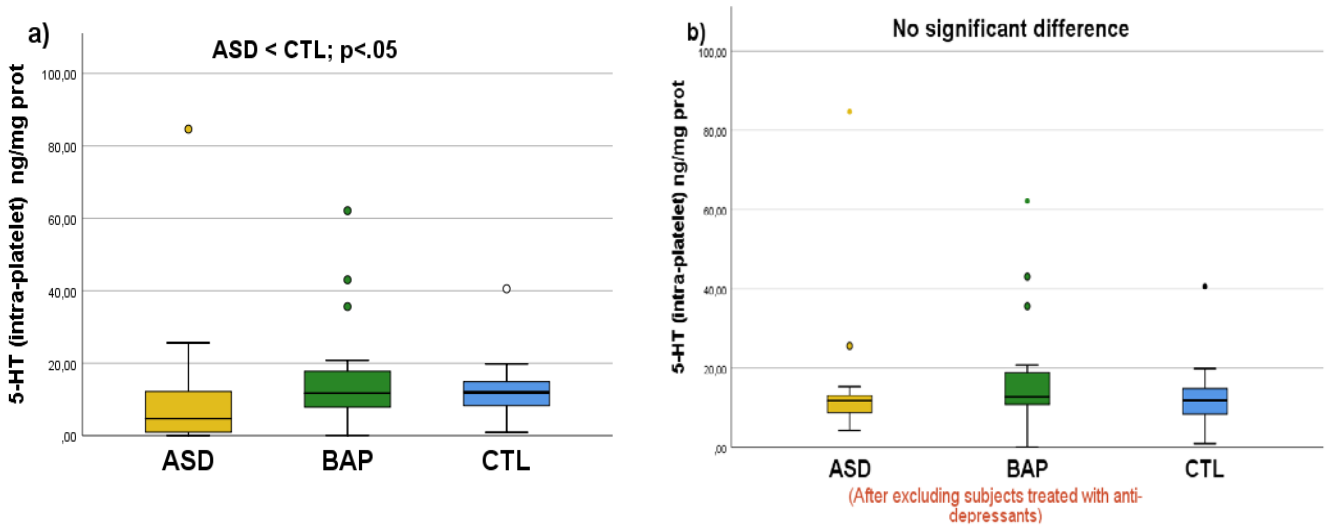

Figure 3R. Comparison of 5-HT (intra-platelet) among groups in the whole sample (3Ra) and after excluding subjects treated with antidepressants (3Rb).

Discussion

While mood/anxiety disorders and/or pharmacological treatments have been reported to influence most of the biochemical parameters evaluated here (Muller et al. 2006; Martinowich and Lu 2008; Dantzer 2009; Harrington et al. 2013; Misiak et al. 2014; Han et al. 2015; Armeanu et al. 2017; Savino et al. 2020; Chengfeng et al. 2019; Sun et al. 2020; Carpita et al. 2020), the absence of significant differences in analyte concentrations depending on other diagnoses and pharmacological treatment within groups, with the exception of intra-platelet 5-HT, may suggest that, in the case of our sample, the impact of the ASD/BAP condition on biochemical parameters' concentration would overcome that of other conditions or of drugs (see also **chapter 5**, Limitations and conclusion).

The overall ranges of concentrations for the biochemical parameters evaluated here reflect those reported in previous studies, although it should be considered the presence of great variability for some parameters in previous research (Tu et al. 2012; Zheng et al. 2017; Lym et al. 2016; Bryn et al. 2017; Ormstad et al. 2018; Gejl et al. 2019; Rehman et al. 2020; Said et al. 2021; Zhao et al. 2021). 5-HT concentrations showed a tendency to be higher in PPP and lower in platelets in our sample than in most of previous studies, although it should be noted that final concentrations of 5-HT in these

specimens are reported to vary greatly on the basis of sample preparation, including centrifugation modalities, and that studies conducted on these kinds of biological samples are particularly heterogeneous with respect to sample preparation and separative procedures as well as to the kind of assay employed (Anderson et al. 1987; Anderson et al. 2012; Gabriele et al. 2014; Von Volkman et al. 2019).

Our results from the comparison of biochemical parameters, while being partially in line with previous literature in children, also suggest some specific patterns of alteration in ASD adults. Regarding 5-HT, we did not find in the patient group the increased levels reported in the literature in around a third of ASD subjects (Gabriele et al. 2014). However, previous research also stated that the increased levels of 5-HT, being reported only in a sub-group of ASD subjects, may not be detectable in smaller samples (Hranilovic et al. 2007; 2009), and, in addition, they seem to be mainly reported in PRP or in whole blood (Gabriele et al. 2014). Moreover, in adult samples hyperserotonemia was reported to be a less stable marker than in children, while an impact of age on 5-HT levels was also hypothesized, with studies reporting lower levels of 5-HT in post-puberal ASD patients than in prepuberal ones (Hranilovic et al. 2007; 2009; Shuffrey et al. 2017; Padmakumar et al. 2019). While most of the studies did not find a clear association between altered levels of 5-HT and specific ASD phenotypes, some authors reported an association of higher 5-HT levels with increased stereotypies/self-injuring or also with more severe communication impairment and altered speech development (Hranilovich et al. 2007; Muller et al. 2016). However, our data showed instead that PPP 5-HT levels in the ASD group were significantly lower when compared with the two other groups. Although the meta-analysis of Gabriele et al. (2014) reported no difference in PPP between ASD and CTL subjects, some previous studies in adult samples did report a lower concentration of 5-HT in PPP among ASD patients (Spivak et al. 2004), showing also an inverse correlation with aggressive behaviors, although this finding was not always confirmed (Anderson et al. 2012). Thus, our data would be in line with these latter researches. Lower concentrations of 5-HT (PPP) in ASD would also be in agreement with the reported beneficial effects of SSRIs (and also of TRP intake) in this population (Spivak et al. 2004; Hollander et al. 2012; Harrington et al. 2013), as well as with the reported worsening of ASD symptoms in response to acute TRP depletion, which, in turn, has been associated with reduced 5-HT synthesis (Savino et al. 2020). Regarding intra-platelet 5-HT, although we found decreased levels in ASD

when evaluating the whole sample, we also found that levels of this parameter may be influenced by the use of antidepressant drugs. In particular, in both ASD and BAP groups, we found significantly lower intra-platelet 5-HT levels in subjects under treatment with antidepressants. The specific effect of SSRIs in reducing intra-platelet levels was already reported in the scientific literature, and our findings are in line with previous research on this matter (Maurer-Spurej 2004). When removing subjects in treatment with antidepressants from the comparison, no significant differences were found among groups, eventually suggesting that, in the case of intra-platelet 5-HT levels, the reduction reported in the ASD group should be accounted to the presence of the pharmacological therapy. This should not instead be the case of 5-HT levels in the PPP, for which subjects in treatment or not in treatment with antidepressants did not significantly differ. Thus, our results seem to globally suggest a reduction of 5-HT levels in the ASD group only in the PPP, but not in platelets - which, at least in physiological conditions, contain the majority of circulating 5-HT. While it is not clear how much circulating 5-HT levels in different biological specimens may reflect the CNS availability (Hranilovic et al. 2009), it would be possible that, as reported in patients with anxiety and depression, the balance between intra- and extra-cellular 5-HT would be altered in ASD (Zhuang et al. 2018). Intriguingly, increased 5-HT uptake by platelets was also reported among ASD subjects (Spivak et al. 2004). As for ASD adults, previous studies regarding the BAP in this field led to controversial results. While some authors reported increased 5-HT levels among parents of ASD probands in the whole blood or also in the PPP (Pagan et al. 2014; Bijl et al. 2015), others (Connors et al. 2006) reported decreased levels of PPP 5-HT in parents of ASD patients, and in particular among mothers, hypothesizing a role of mother's lower 5-HT levels in ASD pathogenesis. However, in our sample, concentrations of 5-HT in the PPP did not significantly differ between BAP and CTL groups. Globally, our results may confirm, as stated by previous authors (Hranilovic et al. 2007; 2009), that the association between ASD and higher levels of 5-HT should be reconsidered as a condition involving in particular the age of neurodevelopment and specific ASD sub-phenotypes. Further studies are needed to clarify which specific features may be associated with increased, reduced or normal levels of 5-HT in this population.

According to most of the previous research in ASD children, which highlighted reduced TRP levels in this population (Zheng et al. 2017), in our sample significantly lower

levels of TRP were reported in the ASD group. In the BAP group, TRP levels were also significantly lower than in CTL subjects, stressing a continuum between patients and relatives for this parameter. To the best of our knowledge, this is the first study involving only adult patients on the KYN pathway in ASD and including also relatives of ASD probands. In this framework, we found that KYNA, as reported in other researches, was significantly decreased in the patient group (Bryn et al. 2017). In line with some previous studies, but in contrast to others, we did not find significant differences in the levels of KYN and QUIN (Lim et al. 2016; Bryn et al.2017). The KYN/TRP ratio, which may reflect IDO activity (Lim et al. 2016), was not different among groups, despite the lower levels of TRP in ASD and BAP subjects. Several factors may have led to reduced TRP and KYNA levels. Although we did not find differences in QUIN concentration, it should be noted that other authors highlighted a higher tendency in ASD children to metabolize TRP also in xanthurenic acid, with a reduction of the KYNA and melatonin routes (Gevi et al. 2016). Among the possible factors associated with lower TRP, besides lower dietary intakes, altered absorption and/or endocrine regulation (Kałużna-Czaplińska et al. 2017), the role of gut microbiota should be also considered. While several microbiota alterations and gut dysbiosis were reported in ASD (Carpita et al. 2020a), microbiota may exert an impact on TRP metabolism in several ways: in particular, Gevi et al. (2016) highlighted its possible role in promoting an increase of indolyl 3-acetic acid through the metabolism of TRP by the indole route by microbiota bacteria (Roth et al. 2021). These findings may allow hypothesizing that in ASD adults the altered TRP metabolism would lead to a depletion of KYNA and a reduction of the KYN pathway towards the neuroprotective branch, while the QUIN branch would remain more conserved, eventually exerting higher excitotoxic effects due to the lack of balance and regulation by the KYNA-related neuroprotective counterpart. Two previous studies in children reported that lower TRP and KYNA levels could be a specific feature of Childhood autism, being eventually linked to intellectual disability, while subjects with Asperger syndrome would show higher TRP levels (Bryn et al. 2017; Ormstad et al. 2018). Our data, reporting lower TRP levels among adult subjects with no intellectual or language impairment, seem not to confirm this result. In this framework, when evaluating the differences between findings in ASD adults and children, it should also be noted that KYN metabolism was reported to be age-dependent, with an association of aging with increased levels of

KYN, KYNA and QUIN (Sorgdrager et al. 2019). Noticeably, the BAP group reported TRP levels similar to those of the ASD group and significantly lower than CTL subjects. However, as for the 5-HT, also KYNA levels in this group were instead not significantly different from CTL. This data may suggest that the metabolism of the BAP group may succeed, eventually through compensatory enzymatic activity, in producing levels of KYNA and 5-HT similar to those of the general population. This hypothesis may be further supported by the higher KYNA/TRP and KYNA/QA reported among BAP when compared with ASD group, this latter ratio eventually reflecting lower levels of excitotoxicity among BAP subjects (Bryn et al. 2017). KYNA/KYN ratio was instead lower in ASD than in CTL subjects, reporting a high variability in BAP group. Bryn et al. (2017) hypothesized that higher KYN/KYNA ratio (or, conversely, lower KYNA/KYN as reported in our study) may suggest a reduced KAT activity, as reported in Alzheimer or Parkinson disease, linked to a higher neurotoxic potential, since KYNA is an antagonist of NMDA receptors. The 5-HT(PPP)/TRP ratio was also increased in the BAP, although without reaching a statistical significance difference from the other groups. Globally, this pattern confirms the relationship between ASD and the impairment of TRP metabolism, which would be present also among relatives, although with milder intensity, eventually implying a lower impact on biochemical functioning in subjects with BAP.

We did not find any difference with respect to BDNF levels. Although, as also confirmed by meta-analytic studies, research in children mostly reported higher BDNF levels in ASD (Quin et al. 2016; Armeanu et al. 2017), studies in this field mainly focused on serum or PRP (Quin et al. 2016). Moreover, it was highlighted in previous literature that the findings related to BDNF increase among ASD children have not been replicated in plasma samples (Quin et al. 2016) and in adults (Zheng et al. 2016). Our results seem thus to be in line with this data. In this framework, Zheng et al. (2016) hypothesized that the BDNF increase reported in ASD may be a feature linked only to early life stages, being involved in ASD pathogenesis (e.g., influencing the alteration of connectivity found in these patients) or being instead a compensatory factor associated with the late brain maturation.

Our results about HCY and IL-6 seem instead to confirm the pattern previously found in ASD children. In our sample HCY concentrations were increased in ASD adults, according to most of previous literature, which highlighted higher levels of HCY in

ASD children or adolescents (Zheng et al. 2017; Guo et al. 2020), although this result was not always replicated (Main et al. 2015; Bala et al. 2016). Noticeably, our sample reported a mean HCY value of 12.939±8.485 μM for the ASD group and of 10.001±3.520 μM for the BAP one, indicating mean values above the optimal ones (which would be below 10 μM) for ASD and borderline values for BAP (Rehman et al. 2020). Moreover, 29.17 % of the ASD group and 8.33 % of the BAP group, but no subject in the CTL group, reported hyper-HCY (values above 15μM). Previous literature showing higher HCY levels in ASD (although focused on children, among which HCY levels are basically lower) alternatively reported, in this population, mean values above or below the threshold of 15 μM (Ali et al. 2011; Tu et al. 2012; Rheman et al. 2020). Increased levels of HCY may be linked to metabolic alterations underlain by genetic factors, but also to nutritional issues, such as insufficient intake or absorption of vitamin B6, B12 or folate, which are crucial for HCY metabolism (Han et al. 2015; Zheng et al. 2017). Reduced DNA methylation and higher oxidative stress associated with altered redox balance and glutathione depletion were previously reported in ASD children (Han et al. 2015; Zheng et al. 2017). These alterations may be associated with impaired methylation and trans-sulfuration pathways of HCY metabolism: thus, HCY may indirectly provide information on DNA methylation and redox state in ASD, besides the impact that HCY can directly exert on these systems (Han et al. 2015; Zheng et al. 2017). In this framework, some of the authors that reported higher HCY concentrations in ASD children also reported lower cysteine, glutathione and GSH/GSSG ratio (Han et al. 2015). In line with the hypothesis of a continuity between ASD and BAP profiles, BAP subjects in our sample reported instead intermediate levels of HCY, being not significantly different from both groups. Previous studies on relatives reported intermediate SAH levels between patients and controls on siblings of ASD children, although they did not find differences with respect to HCY (Melnyk et al. 2012). On the other hand, James et al. (2008; 2010) reported higher levels of HCY, SAH, GSSG and lower levels of GSH, GSH/GSSG and SAM/SAM ratio among parents of ASD probands, together with DNA hypo-methylation, when compared with parents of non-affected children. Globally, our findings seem to confirm also in adults an association of the autism spectrum with altered HCY levels, which are in turn potentially linked to altered methylation/trans-sulfuration pathways (Melnyk et al. 2012; Han et al. 2015).

Similar results were found for IL-6, which in our sample was significantly increased in ASD patients with respect to CTL subjects. Previous studies in the field of ASD mostly reported in children increased pro-inflammatory and decreased anti-inflammatory cytokines (Carpita et al. 2020a). IL-6 is commonly considered as a pro-inflammatory cytokine, although it may also exert anti-inflammatory effects (Gadient and Otten 1997), and it is one of the cytokines most constantly reported increased in ASD children, in both the periphery and the CSF, according to meta-analytic studies (Masi et al. 2015; Carpita et al. 2020a; Zhao et al. 2021). Some studies in small samples of adult ASD subjects did not find significant differences between patients and controls with respect to IL-6, despite finding a non-significant trend towards an increase of IL-6 among ASD patients (Chroonenberghs et al. 2002b; Emanuele et al. 2010). In the BAP group, we found intermediate levels, not significantly different from the other two groups: previous studies on relatives also reported no differences between ASD and their siblings (Napolini et al. 2013), while one study reported lower IL-6 levels in ASD children than in siblings of other ASD patients (Manzardo et al. 2012). Several cytokines, including IL-6, were also reported to be higher in pregnant mothers of ASD patients than in control mothers (Jones et al. 2017; Carpita et al. 2020). In CNS, IL-6, which is also able to affect the KYN pathway (Savino et al. 2020), seems to be involved in the promotion of sickness behavior (Gadient and Otten 1997; Dantzer 2009). While IL-6 may also exert a positive action in regulating neuronal survival and differentiation, its over-expression was linked to neurodegeneration (Gadient and Otten 1997; Dantzer 2009). Besides other psychiatric disorders, neurodegenerative disorders, such as Alzheimer's disease, or autoimmune disorders, such as multiple sclerosis, were also associated with an increase of IL-6 (Gadient and Odent 1997). IL-6 is supposed to play a crucial role in mediating the communication between CNS and immune system: the increased levels reported in our sample among ASD patients may confirm the pattern of altered immune system/inflammatory activity in ASD stressed in previous literature, and the possible reciprocal interactions between CNS and immune system in shaping their activities (Carpita et al. 2020a).

4.3 Correlations among biochemical parameters

Results

When evaluating the Spearman correlation coefficient among biochemical parameters in the whole sample, we found that KYNA levels were significantly and positively correlated with KYN and QUIN concentrations. KYN and QUIN levels were also positively correlated with each other. A significant negative correlation was instead found between KYNA and HCY levels. Finally, 5-HT levels in PPP and intra-platelet 5-HT were positively correlated (see **Table 6** and **Figure 4R**)

	HCY, µM	IL-6, pg/mL	BDNF, ng/mL	QUIN, ng/mL	KYNA, ng/mL	KYN, ng/mL	TRP, µM	5-HT (platelet), ng/mg proteins	5-HT (PPP), ng/mL
5-HT(PPP), ng/mL	-.207	-.065	.144	-.053	.051	-.197	.039	**.341****	-
5-HT(platelet), ng/mg proteins	-.109	-.165	.179	.097	.138	-.124	.036		**.341****
TRP, µM	-.110	-.119	-.138	-.029	.039	-.034	-	.036	.039
KYN, ng/mL	.102	.077	-.160	**.564****	**.337****	-	-.034	-.124	-.197
KYNA, ng/mL	**-.267***	-.235	-.014	**.267***	-	**.337****	.039	.138	.051
QUIN, ng/mL	.071	-.077	-.081	-	**.267***	**.564****	-.029	.097	-.053
BDNF, ng/mL	-.031	-.010	-	-.081	-.014	-.160	-.138	.179	.144
IL-6, pg/mL	.291	-	-.010	-.077	-.235	.077	-.119	-.165	-.065
HCY, µM	-	-.291	-.031	.071	**-.267***	.102	-.110	-.109	-.207

Table 6. Correlations (Spearman r) between biochemical parameters in the whole sample (n = 72).

**p<.05; **p<.01*

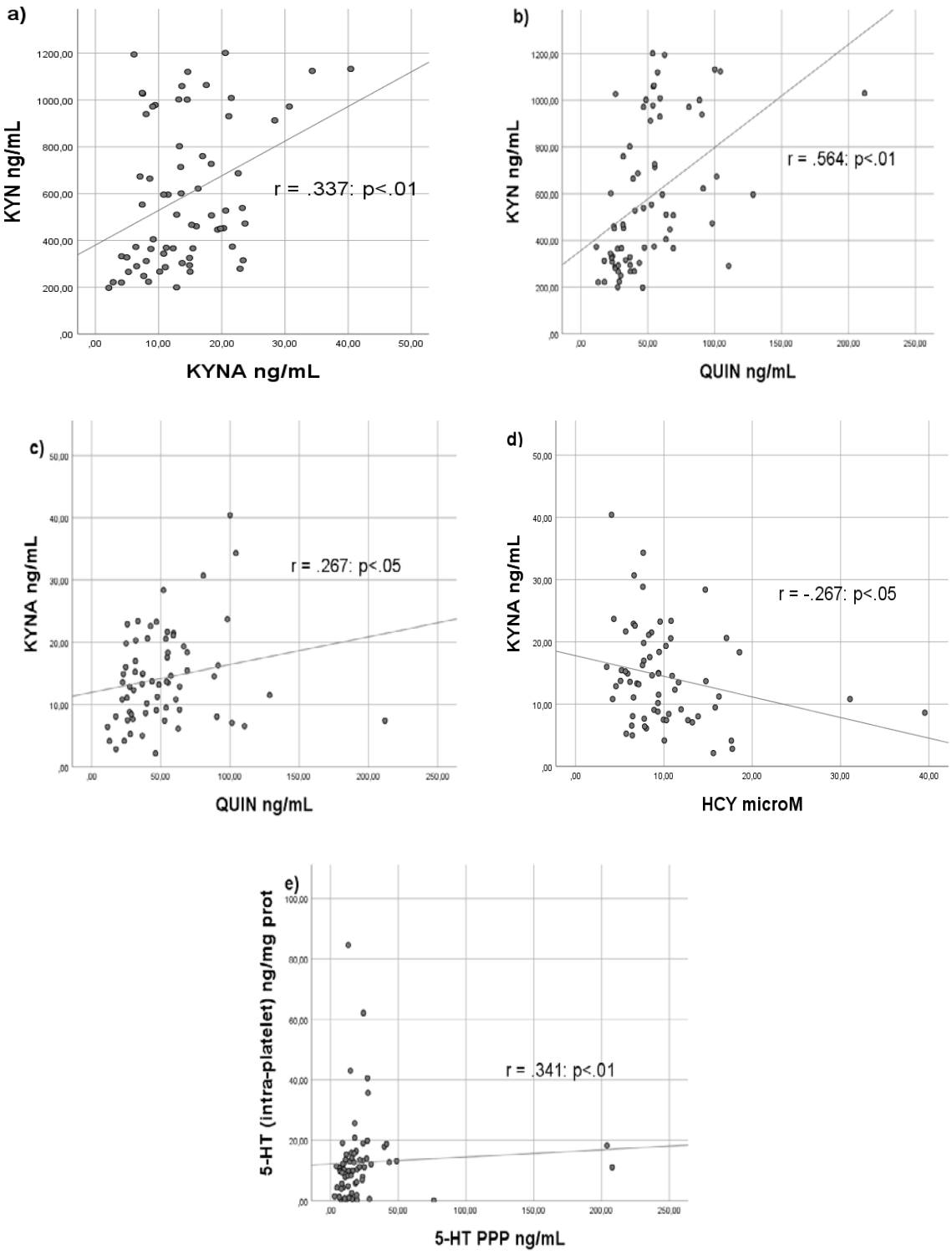

Figure 4R. Significant correlations among biochemical parameters in the whole sample (n = 72) with Spearman r coefficient and p value. 4Ra) KYN vs. KYNA; 4Rb) KYN vs. QUIN; 4Rc) KYNA vs. QUIN; 4Rd) KYNA vs. HCY; 4Re) 5-HT (intra-platelet) vs. 5-HT (PPP).

We performed a further analysis in order to evaluate the correlations between intra-platelet 5-HT levels and the other parameters in subjects who were not taking antidepressants. We found that, in this case, intra-platelet 5-HT levels were positively correlated not only with 5-HT levels in PPP, but also with BDNF (see **Table 7**).

	5-HT (platelet), ng/mg proteins
5-HT(PPP), ng/mL	**.329***
TRP, µM	-.044
KYN, ng/mL	-.155
KYNA, ng/mL	-.011
QUIN, ng/mL	.034
BDNF, ng/mL	**.274***
IL-6, pg/mL	-.110
HCY µM	-.058

Table 7. Correlations (Spearman r) between intra-platelet levels of 5-HT and the other biochemical parameters among subjects not in treatment with antidepressants (n = 58).

**p<.05; **p<.01*

Discussion

Considering the correlations carried out among the parameters involved in TRP metabolism, we found significant positive correlations among parameters of the same route (within KYN or within 5-HT pathway), but we did not find any correlation between parameters of KYN pathway and 5-HT, eventually suggesting that the alteration of one route may be independent by the impairment of the other one, at least at the peripheral level. The correlations found between intra-platelet 5-HT and BDNF may instead confirm the reciprocal interactions between these two analytes, which are

known to regulate and promote the expression of each other (Martinowich and Lu 2008). On the other hand, we found an inverse correlation between KYNA and HCY levels. This result may be considered in line with previous studies, which highlighted an effect of HCY in modulating KYNA levels. While some studies reported a positive correlation between these two parameters, hypothesizing a possible protective role of KYNA in contrasting the harmful effects mediated by HCY, a deeper investigation reported a biphasic modulation of KYNA by HCY in the brain (Luchowska et al. 2005; Wejksza et al. 2009; Pawlak et al. 2012). In particular, while low HCY concentrations seemed to promote KYNA production, higher HCY concentrations seemed to inhibit KYNA synthesis, by the inhibition of KAT activity (Luchowska et al. 2005; Urbańska et al.2006). These researches led authors to also hypothesize that brain impairment associated with higher HCY may be partially mediated by lower KYNA levels (Luchowska et al. 2005).

4.4 Multinomial logistic regression analysis

Results

A multinomial logistic regression analysis was performed in order to evaluate which biochemical parameters were most statistically predictive of being in the ASD or the BAP group. The group category was the dependent variable (with CTL group as the reference category) and the independent variables were 5-HT in PPP, KYNA, TRP, HCY and IL-6 levels, since these were the biochemical parameters for which significant differences were found among groups. Results showed that higher levels of HCY and IL-6 were statistically predictive of being in the ASD group, while only higher levels of IL-6 were statistically predictive of being in the BAP (see **Table 8** and **figure 5R**).

		B(SE)	Odds ratio	CI (95%)		p
				Upper limit	Lower limit	
ASD	**Intercept**	-9.07(5.58)	-	-	-	.104
	5-HT(PPP), ng/mL	-.060(0.07)	0.94	0.82	1.09	.405
	TRP, µM	-0.10(0.05)	0.91	0.82	1.00	.056
	KYNA, ng/mL	-0.07(0.41)	0.936	0.733	1.196	.598
	IL-6, pg/mL	0.60(0.29)	1.83	1.04	3.22	**.036**
	HCY, µM	0.92(0.41)	2.50	1.12	5.60	**.026**
BAP	**Intercept**	-.839(5.48)	-	-	-	.126
	5-HT(PPP), ng/mL	-0.06(0.07)	0.94	0.82	1.10	.421
	TRP, µM	-0.09(0.95)	0.91	0.83	1.00	.060
	KYNA, ng/mL	-0.03(0.12)	0.97	0.77	1.22	.785
	IL-6, pg/mL	0.60(0.29)	1.82	1.04	3.19	**.036**
	HCY, µM	0.79(0.41)	2.20	0.99	4.86	.052

R2(Cox/Smell)=.589; R2(Nagelkerke)=.663; Model fitting: Chi-square(10)=32.90; p<.001

Table 8. Multinomial logistic regression analysis with "group" as the dependent variable (CTL group as reference category) and 5-HT (PPP), KYNA, HCY, IL-6 as the independent variables.

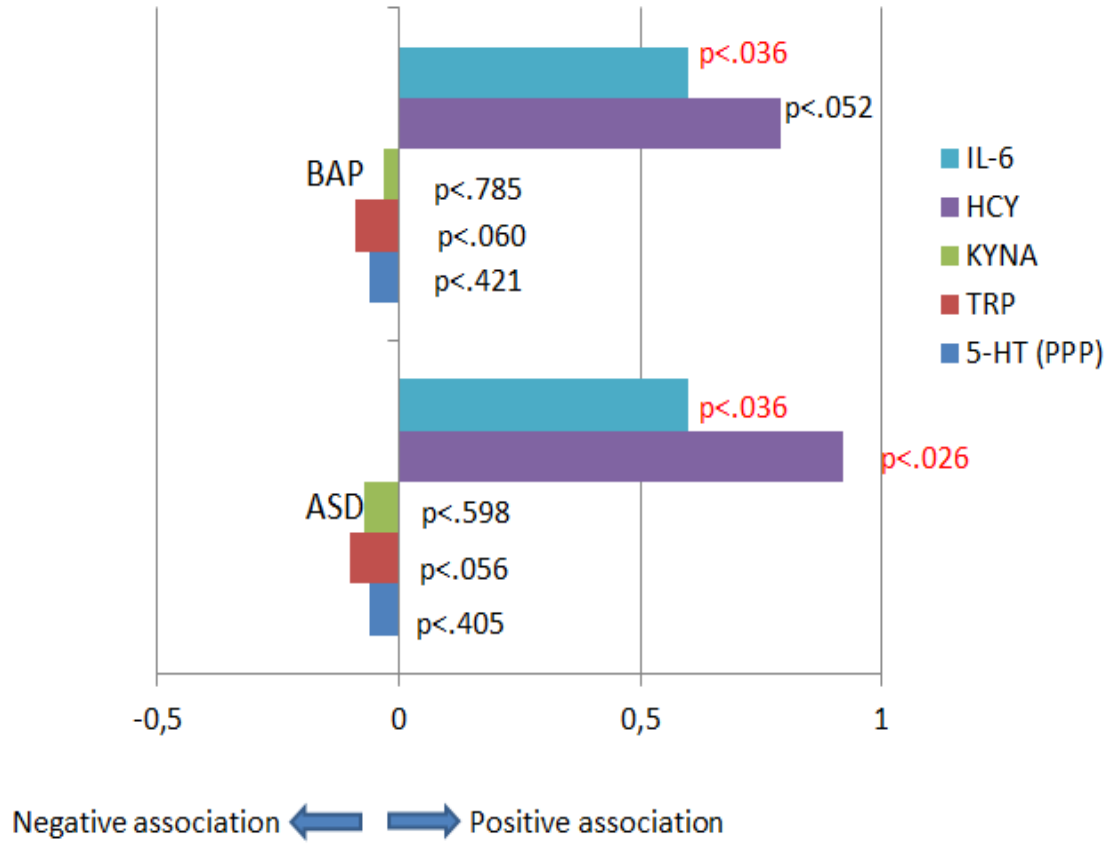

Figure 5R. Schematic representation of the independent variables statistically predictive of being in the BAP or in the ASD group (with CTL group as reference category) according to the multinomial logistic regression model (see also Table 8).

Discussion

Among all the parameters reported to be altered in the previous analyses, results from the regression model seemed to identify in the increase of IL-6 and HCY the statistically predictive variables of ASD. This model highlighted and attributed more importance to the IL-6 increase also in the BAP, suggesting an association of BAP with immune system alteration, possibly with a lower impairment of redox balance and

methylation processes eventually associated with HCY increase. However, it should be noted that also in BAP group a trend toward a statistically predictive value of HCY was observed (p = .052), while in both ASD and BAP group there was a trend towards a negative predictive value of TRP levels (p = .056 and p = .060 respectively). Although it is possible that the association with TRP (and with HCY for the BAP group) would have reached the statistical significance in a wider sample, our results may still imply a greater association of IL-6 and HCY alterations with the autism spectrum. Globally, these data suggest a significant role of immune system/inflammatory alteration, and, on the other hand, of oxidative stress and altered methylation and trans-sulfuration processes in ASD, that seem to overcome the alteration of 5-HT and KYN systems in their association with ASD. In this framework, it should also be noted how both HCY and IL-6 levels seem to be able to modulate TRP metabolism and or KYN pathway, suggesting the involvement in ASD pathophysiology of intertwined relationships between these different systems (Luchowska et al. 2005; Melnyk et al.2012; Han et al. 2015; Carpita et al. 2020a; Savino et al. 2020).

4.5 Correlations between biochemical parameters and psychometric instruments

Results

Finally, we evaluated the presence of significant correlations among biochemical parameter concentrations and scores reported on psychometric scales. As described in the method section, for all the scales employed higher scores are associated with a higher impairment in the investigated dimension. The AdAS Spectrum total score was significantly and negatively correlated with TRP, KYNA and PPP 5-HT levels, while it was positively correlated with HCY concentrations. All AdAS Spectrum domains were negatively correlated with KYNA levels (with exception of *Empathy* domain), TRP levels (with the exception of *Childhood/adolescence* domain) and 5-HT (PPP) levels (with the exception of *Hyper-hyporeactivity to sensory input* domain). Moreover, a significant positive correlation was found between HCY and all AdAS Spectrum domain, with the exception of *Empathy* (see **Table 9a**).

	Childhood /adolescence	*Verbal commun*	*Non-verbal commun*	*Empathy*	*Inflexibility and adherence to routine*	*Restricted interests and rumination*	*Hyper-hypo reactivity to sensory input*	*AdAS Total score*
5-HT (platelet), ng/mg proteins (no antidep)	.133	.111	.118	.004	.036	.189	.006	.115
5-HT (PPP), ng/mL	**-.274***	**-.314***	**-.380****	**-.296***	**-.325****	**-.311***	-.233	**-.369****
5-HT (platelet), ng/mg proteins	-.212	-.154	-.192	-.185	-.182	-.161	-.201	-.205
TRP, µM	-.174	**-.316***	**-.259***	**-.290***	**-.259***	**-.252***	**-.350****	**-.300***
KYN, ng/mL	-.085	-.147	.005	-.013	-.093	-.113	-.006	-.059
KYNA, ng/mL	**-.314***	**-.366***	**-.344****	-.166	**-.362****	**-.444****	**-.322****	**-.362****
QUIN, ng/mL	-.160	-.077	-.009	-.024	-.053	-.021	-.026	-.067
BDNF, ng/mL	-.103	-.030	-.186	-.144	-.050	-.031	.008	-.128
IL-6, pg/mL	.229	.202	-294	.175	.322	.284	.235	.269
HCY, µM	**.332****	**.260***	**.274***	.202	**.345****	**.385****	**.280***	**.345****

Table 9a. Correlations (Spearman r) between biochemical parameters and AdAS Spectrum scores in the whole sample (n = 72).

**p<.05; **p<.01*

Considering the AQ, we found significant correlations involving the same parameters which were significantly associated with the AdAS Spectrum, although correlations with the AQ were more scattered and globally fewer number. In particular, the AQ total score was negatively correlated with 5-HT (PPP) and KYNA levels, and positively with HCY levels (see **Table 9b**). HCY levels were also positively correlated with the AQ *Social skill* domain. 5-HT (PPP) levels were instead negatively correlated with *Attention switching* and *Communication* domains. This latter was the domain which showed more correlations with biochemical parameters, reporting also a negative correlation with TRP and KYNA levels and a positive one with IL-6.

	Social skill	*Attention switching*	*Attention to detail*	*Communication*	*Imagination*	*AQ total score*
5-HT (platelet), ng/mg proteins (no antidep)	.214	.014	-.121	.043	.013	.086
5-HT (PPP), ng/mL	-.151	**-.451****	-.177	**-.353****	-.048	**-.331***
5-HT (platelet), ng/mg proteins	-.154	-.249	-.161	-.173	-.129	-.247
TRP, µM	-.155	-.078	-.032	**-.272***	.006	-.255
KYN, ng/mL				.142	-.086	
KYNA, ng/mL	-.248	-.240	-.267	**-.300***	-.097	**-.398****
QUIN, ng/mL	-.093	.005	-.029	.066	-.198	-.147
BDNF, ng/mL	.052	-.015	.080	-.017	.032	.044
IL-6, pg/mL	.247	.288	.316	**.428***	.070	.353
HCY, µM	**.269***	.195	.147	.230	.171	**.368****

Table 9b. Correlations (Spearman r) between biochemical parameters and AQ scores in the whole sample (n = 72).

**p<.05; **p<.01*

When exploring correlations between biochemical parameters and RAADS-14, we found similar results than those reported for the other scales for the total scores, with RAADS-14 total score being negatively correlated with 5-HT in PPP, TRP and KYNA levels and positively with HCY levels. While 5-HT levels in the PPP and KYNA levels were significantly (and negatively) correlated with all RAADS-14 domains, for TRP a significant negative correlation was found only for the *Sensory reactivity* domain. HCY was instead positively correlated with *Mentalizing deficit* and *Social anxiety* domains. Moreover, the *Mentalizing deficit* domain was also significantly and positively correlated with IL-6 levels. Finally, a significant positive correlation was found between intra-platelet 5-HT levels and the *Sensory reactivity* domain, but only after excluding subjects in treatment with antidepressants (see **Table 9c**).

	Mentalizing deficit	*Social anxiety*	*Sensory reactivity*	*RAADS-14 Total score*
5-HT (platelet), ng/mg proteins (no antidep)	.094	.141	**.394****	.236
5-HT (PPP), ng/mL	**-.399****	**-.357****	**-.299***	**-.401***
5-HT (platelet), ng/mg proteins	-.249	-.230	.079	-.160
TRP, µM	-.228	-.197	**-.281***	**-.275***
KYN, ng/mL	.053	-.033	-.065	-.005
KYNA, ng/mL	**-.344****	**-.336****	**-.266***	**-.331****
QUIN, ng/mL	-.040	-.093	-.100	-.079
BDNF, ng/mL	-.004	.012	.102	.082
IL-6, pg/mL	**.346***	.124	.179	.267
HCY, µM	**.392****	**.280***	.216	**.358****

Table 9c. Correlations (Spearman r) between biochemical parameters and RAADS-14 scores in the whole sample (n = 72).

**p<.05; **p<.01*

RRS total and all domain scores, which measure the different dimensions of ruminative thinking, were significantly and negatively correlated with TRP and KYNA levels, and positively correlated with IL-6 and HCY levels, with the only exception, for HCY, of the *Reflection* domain (see **Table 9d**).

	Reflection	*Brooding*	*Depression*	*RRS Total score*
5-HT (platelet), ng/mg proteins (no antidep)	.181	.179	.234	.222
5-HT (PPP), ng/mL	-.134	-.189	-.254	-.242
5-HT (platelet), ng/mg proteins	-.112	-.095	-.193	-.173
TRP, µM	**-.398****	**-.351****	**-.394****	**-.407****
KYN, ng/mL	.056	.013	-.035	-.027
KYNA, ng/mL	**-.262***	**-.253***	**-.351****	**-.336****
QUIN, ng/mL	-.081	-.091	-.034	-.057
BDNF, ng/mL	.140	.044	.135	.107
IL-6, pg/mL	**.409***	**.475****	**.509****	**.483****
HCY, µM	.257	**.469****	**.422***	**.403****

Table 9d. Correlations (Spearman r) between biochemical parameters and RRS scores in the whole sample (n = 72).

**p<.05; **p<.01*

Finally, we evaluated the correlations between biochemical parameters and the WSAS, which explores the negative impact of symptoms on different areas of functioning (the higher the score, the higher the negative impact) (see **Table 9e**). We found that WSAS total scores were negatively correlated only with PPP 5-HT levels, which were, in turn, negatively correlated also with the items exploring *Work* and *Home management* related functioning. The item exploring *Close relationships* was instead negatively correlated with TRP levels. Moreover, the WSAS total score and almost all single item scores were positively correlated with IL-6 (with the exception of *Home management*) and HCY (with the exception of *Social leisure activities* and *Close relationships*).

	Work	*Home management*	*Social leisure activities*	*Private leisure activities*	*Close relationships*	***WSAS total score***
5-HT (platelet), ng/mg proteins (no antidep)	.037	-.002	.113	.102	.010	.095
5-HT (PPP), ng/mL	**-.345****	**-.311***	-.168	-.191	-.197	**-.260***
5-HT (platelet), ng/mg proteins	-.206	-.245	-.207	-.230	-.129	-.207
TRP, µM	-.166	-.148	-.183	-.149	**-.271***	-.167
KYN, ng/mL	-.066	-.055	-.012	-.051	-.082	-.063
KYNA, ng/mL	-.228	-.191	-.150	-.207	-.203	-.242
QUIN, ng/mL	.007	-.067	-.100	-.060	-.123	-.063
BDNF, ng/mL	.035	.115	.145	.132	.123	.116
IL-6, pg/mL	**.432***	.260	**.406***	**.548****	**.489****	**.477****
HCY, µM	**.317***	**.365****	.230	**.268***	.242	**.326***

Table 9e. Correlations (Spearman r) between biochemical parameters and WSAS scores in the whole sample (n = 72).

**p<.05; **p<.01*

<u>*Discussion*</u>

Globally, the significant correlations reported here involved the same biochemical variables which resulted significantly altered also in the comparisons among groups, confirming their association with autism-related psychopathology.

Concentrations of 5-HT (PPP), TRP and KYNA showed significantly negative correlations with most of the autistic dimensions, with some differences in the pattern of

association with specific clusters of symptoms. In particular, 5-HT (PPP) reported the highest negative correlations with the dimension of social communication deficits and social anxiety, inflexibility and impairment in attention switching (the highest correlation for 5-HT), with no association with the ruminative dimension as measured by the RRS. Intriguingly, there was a unique positive correlation between 5-HT (intra-platelet) levels and the presence of higher sensory reactivity (as measured by the RAADS), while 5-HT (PPP) was inversely correlated with the same dimension. TRP and KYNA were negatively associated with a greater impairment in social communication and altered reactivity/sensitivity to sensorial stimuli, although they showed their highest negative correlations with the ruminative dimension, as measured by the RRS and, in particular for KYNA, also by the AdAS Spectrum. Previous literature has reported positive associations between 5-HT levels and behaviors such as self-injuring or stereotypies among ASD subjects, but 5-HT relationships with other autistic dimensions were poorly investigated (Hranilovic et al. 2009; Muller et al. 2016). In the framework of social communication and interactions, a negative correlation between aggressive behavior and 5-HT (PPP) was reported by one study in adults, but none of our questionnaires specifically investigated this dimension (Spivak et al. 2004). Hranilovic et al. (2007) reported that higher levels of 5-HT were correlated with poorer speech development and verbal abilities. This result is not necessarily in contrast with our findings, because 5-HT levels were measured in platelets and not in PPP. On the other hand, our results are in line with the improvement in language skills, social functioning and repetitive behaviors reported with SSRI treatment among ASD subjects (DeLong et al. 1998; Hollander et al. 2012; Harrington et al. 2013). Moreover, several studies, which might be considered in line with our findings, highlighted the crucial role of 5-HT in pro-social behaviors and social communication, while other investigations showed that lower 5-HT in the CNS was associated with communication impairment in rats (Kiser et al. 2012; Beis et al. 2015). In addition, SSRIs are also approved drugs for Social anxiety disorder, and are reported to improve interpersonal behavior and perception in this population (Rapport et al. 2018). According to our findings about the involvement of TRP in the communication area, previous studies highlighted that, in rats, social behaviors were improved by TRP dietary supplement, and worsened by TRP depletion (Zhang et al. 2015; 2017). In humans, dietary deprivation of TRP was reported to worsen ASD symptoms, mainly in repetitive behaviors/stereotypies

(McDougle et al. 1996; Lim et al. 2016; Savino et al. 2020). Moreover, a specific link between TRP and memory-related functions was reported, with a worsening of episodic memory for verbal information after TRP depletion (Mendelsohn et al. 2009; Savino et al. 2020), while, in turn, verbal memory plays a crucial role in language and communication abilities (Tyson et al. 2014). As in the case of 5-HT, TRP supplementation was reported to reduce aggressive behavior, globally confirming the involevement of these two molecules in the social brain (Savino et al. 2020). Regarding KYNA, scant literature is available on its association with specific clusters of symptoms, in particular in the field of ASD. Some studies in Schizophrenia stressed an association of cognitive inflexibility with increased KYNA levels in the CSF (possibly associated with lower circulating levels), or, alternatively, with increased plasmatic levels, but results remain controversial (Bilgiç et al. 2020; Marx et al.2020; Huang et al.2020; Hebbrecht et al. 2021). Other authors are instead stressing the role of TRYCAT metabolism in modulating anxiety not only by causing 5-HT depletion but also by acting as endogenous anxiolytics or anxiogens, including on the basis of their mechanism as NMDA agonists or antagonists: in this framework, KYNA may exert an anxiolytic effect (Kim and Jeon 2018). The association between greater impairment in social communication and lower KYNA levels found in our study may be somewhat in line with the presence of decreased KYNA levels and of unbalanced neurotoxic/neuroprotective branches of the KYN pathway observed in social isolation rearing models of rats (Möller et al. 2012).

The correlation between higher impairment in attention switching and lower 5-HT (PPP) levels reported herein is in line with previous studies that stressed the role of 5-HT in modulating attention and cognitive flexibility, as well as with studies reporting impaired cognitive flexibility in both animal and human models of 5-HT depletion, including acute TRP depletion (Evers et al. 2003; Clarke et al. 2004; 2005; Weinberg-Wolf et al. 2018). According to our results about a link of 5-HT with increased response to sensory stimuli, 5-HT was previously reported to play an important role in the development of sensory functions and in sensory processing, although through mechanisms still unclear (Waterhouse et al. 1986; Sienmann et al. 2017). Noticeably, in mouse models, a gain-of-function SERT variant, linked to a greater 5-HT uptake, was associated with a lower ability to process stimuli and sensory aversion (Waterhouse et al. 1986; Sienmann et al. 2017). This data may be eventually in line with our findings of

a specific association of greater sensory sensitivity with increased 5-HT (intra-platelet) levels, but also with lower 5-HT (PPP) ones. TRP and, to a lesser extent, KYNA, also showed significantly negative correlations with altered sensory reactivity (in this case, including both the hypo-and the hyper-reactivity, as measured by the AdAS Spectrum): this result may be considered in accordance with previous literature which reported altered processing of different stimuli, such as taste or pain perception, in TRP depletion models (Martin et al. 2017; Smith et al. 2021). It should be noted that, on the basis that acute TRP depletion seems to reduce 5-HT synthesis, acute TRP depletion models were employed for investigating the effects of reduced 5-HT levels in humans in different fields, assuming that TRP depletion effects should be mainly attributed to their negative impact on 5-HT production, eventually including their action on memory, social behavior or response to stimuli. On the other hand, as previously described in the introduction section, TRP metabolic activity is not limited to 5-HT production and thus the effects of acute TRP depletion should not necessarily been associated only with the alteration of 5-HT levels (van Donkelar et al. 2011; Kim and Jeon 2018; Savino et al. 2020). As described above, the highest negative correlations for TRP and KYNA were reported with the dimension of rumination, which is a maladaptive, repetitive kind of thinking not leading to active problem solving but to an over-fixation on the problem and on the feelings related to it. Among ASD subjects, ruminative thinking was hypothesized to be involved in the impaired adjustment to stressful events; however, rumination was also considered a trans-nosographic dimension, eventually underlain by the presence of sub-threshold autistic traits, and it was associated with a higher vulnerability towards developing psychiatric symptoms after stressful events and with a higher suicidality risk (Nolen-Hoeksema et al. 2008; Dell'Osso et al. 2019a; 2019c; 2020b). Noticeably, while no study specifically investigated the association of ruminative thinking with these biochemical variables, a specific effect of ketamine, which, as KYNA, is an antagonist of NMDA receptor, was reported in reducing ruminative thinking in psychiatric disorders, also among those resistant to SSRI treatment, eventually suggesting a role of glutamatergic transmission in ruminative thinking (Lehmann et al. 2016; Vidal et al. 2020; Karthik et al. 2020). On the other hand, in agreement with the lack of association between RRS and 5-HT concentrations in the present work, the efficacy of SSRI treatment in reducing repetitive thoughts in neurodevelopmental disorders was reported to be controversial (McDougle et al. 2000).

In the case of HCY we found that the highest correlations (in this case positive ones) were reported with the ruminative dimension as measured by RRS (in particular for the more emotionally negative kinds of rumination: brooding and depressive rumination), and subsequently with inflexibility traits. Positive correlations with social communication impairment were present but were globally weaker with respect to those reported for the previous parameters, while HCY seemed instead more correlated with the broader dimension of social skills. Partially in accordance with these results, previous literature in the field of ASD reported a correlation between impaired communication skills and higher HCY among ASD children (Puig-Alcaraz et al. 2015) and a global correlation with the severity of autistic symptoms, as measured by other psychometric scales mostly used on children and/or in severe forms of ASD, such as the CARS and the ABC (Han et al. 2015).

Moreover, a positive correlation between HCY levels, cognitive performance and hippocampal volume was also reported in the general population, stressing the possible impact of HCY on behavioral and cognitive features (Budge et al. 2002; Barnea-Goraly et al. 2014; Han et al. 2015). Noticeably, according to the present findings, previous studies reported that both higher HCY and lower folate levels were associated with cognitive impairment and inflexibility (Moustafa et al. 2014; Eszlari et al. 2016). In addition, a specific correlation was reported between ruminative thinking and the A allele of a polymorphism (*rs11754661*) of the methylenetetrahydrofolate dehydrogenase 1 like (MTHFD1L) gene: MTHFD1L, also known as 10-formyl-tetrahydrofolate synthetase, catalyzes the transformation of formyl-tetrahydrofolate to formate and tetrahydrofolate (THF): the *A* allele is associated with altered enzymatic function and higher levels of HCY. Globally, our results provide support to the involvement of HCY in the pathophysiology of ruminative thinking. Higher HCY levels may affect cognitive functions and promote ruminative thinking in several ways, such as increasing oxidative stress, impairing methylation processes or also by acting as an agonist of NMDA receptors (Moustafa et al. 2014). In this framework, it should be noted how we found instead an inverse correlation between ruminative thinking and the levels of KYNA (which is a NMDA antagonist), as well as an inverse correlation between KYNA and HCY. As reported above, the possible biphasic modulation of KYNA levels by HCY, with an inhibition of KYNA synthesis by higher HCY levels, was highlighted in previous literature (Luchowska et al. 2005; Urbańska et al. 2006), with authors

hypothesizing that the reduction of KYNA might be at least partially involved in the altered cognitive mechanism linked to higher HCY levels (Luchowska et al. 2005).

A quite different pattern of associations was found for IL-6, for which no correlation was reported for the total scores of the scales measuring the whole autistic symptomatology (AdAS Spectrum, AQ, RAADS). IL-6 was instead found positively correlated only with specific autistic dimensions, such as communication impairment, as globally measured by the AQ (without the separation of verbal/non-verbal areas) and with all dimensions of ruminative thinking as measured by the RRS, for which the correlations reported with IL-6 were stronger than those reported with the other parameters. Given the involvement of IL-6 in several aspects of CNS pathophysiology, including the promotion of sickness behavior (Dantzer 2009), previous research stressed the possible role of IL-6 in modulating autistic-like symptoms, including social withdrawal and cognitive alterations (Wei et al. 2012a; 2012b; 2013). Cognitive deficits were also reported in *IL-6/IL-4 knockout* mouse models (Carpita et al. 2020). Previous studies in ASD children reported an association of communication impairment with cytokine levels but not specifically with IL-6, which seemed instead more associated with repetitive behaviors (Ashwood et al. 2011a; 2011b; Carpita et al. 2020a). Moreover, increased IL-6 levels were reported to be associated with poorer sleep quality (Friedman et al. 2005; Rohleder et al. 2012; Masi et al. 2015) and also by poorer social relationships (Friedman et al. 2005; Eisenberg et al. 2017). These results were partially confirmed by the correlation between communication impairment and higher IL-6 reported in the present work. Noticeably, some authors previously stressed how the relationship between social impairment and IL-6 should be considered bi-directional: while IL-6 may alter social behavior and cognition, including through the modulation of social-related feedback, social isolation or social stress may instead increase pro-inflammatory activity and IL-6 levels (Eisenberg et al. 2017). On the other hand, while the involvement of IL-6 in promoting cognitive function alteration and depressive mood may also be implicated in the pathophysiology of ruminative thinking, it was hypothesized that ruminative thinking may enhance inflammatory processes, eventually increasing IL-6 levels (Moriarity et al. 2018). Moriarity et al. (2018), investigating a sample of adolescents by means of moderated mediation analyses, reported that a higher level of rumination might be a risk factor for the development of higher IL-6 concentrations, which, in turn, may lead to anxiety symptoms and subsequently

depressive symptoms. Another study reported instead that neutral, not maladaptive, reflection may be related to lower IL-6 levels (Woody et al. 2016). Globally, our findings are in agrrement with the relationship between ruminative thinking and IL-6 suggested by previous literature. In this framework, it should be noted that also the association, reported in previous studies, between altered sleep-waking cycles/poor sleep quality and increased IL-6 levels might be considered bi-directional (Friedman et al. 2005; Rohleder et al. 2012; Masi et al. 2015). Moreover, broadening the picture, altered sleep and circadian rhythms have been associated with a higher tendency towards ruminative thinking, which has been hypothesized to moderate the relationship between psychological stress and insomnia (Benham 2021).

In conclusion, according to our results the biochemical variables more correlated with the severity of general autistic symptoms as measured by the psychometric scale total scores (AdAS Spectrum, AQ, RAADS-14) were 5-HT (PPP), KYNA (negative correlations) and HCY (positive correlations), followed by TRP (negative correlations), while the ruminative thinking dimension, as measured by RRS, was more associated with higher levels of HCY and, to a even greater extent, of IL-6. In the case of the impact on work and social functioning as measured by the WSAS, we found instead that the strongest (positive) correlations occurred with IL-6 and HCY, followed by a weaker negative correlation with 5-HT (PPP), which seemed to affect mainly the work/home management areas. Globally, these results may suggest that lower 5-HT (PPP), KYNA and TRP levels would be more closely associated with the presence of the typical core of autistic-like symptoms and traits (as a whole and featuring the above mentioned patterns of association with specific dimensions), but provide less information on the actual impact of symptoms on subjects' life. On the other hand, the greater levels of distress and functional impairment linked to the presence of autistic-like symptoms would be associated with higher levels of HCY and IL-6, with the latter also appearing specifically associated with the ruminative thinking and eventually to the communication impairment dimension. On the basis of this result, it might be hypothesized that subjects' functional impairment and higher inflammatory/oxidative state and/or altered methylation processes may be the reflection of each other, as previously suggested for aging (Martin and Grotewiel 2006; Sartori et al. 2012). These results may also support the hypothesis of a crucial involvement of ruminative thinking in worsening global functioning (Nolen-Hoeksema et al. 2008; Dell'Osso et al. 2019a;

2019c; 2020b). On the other hand, as previously reported, the pathophysiology of autistic symptoms may include the key contribution of an impaired 5-HT network and KYN pathway, as well as of HCY-related metabolism and immune system alterations, eventually with intertwined interactions and different impacts on specific clusters of symptoms (Luchowska et al. 2005; Han et al. 2015; Bryn et al. 2017; Savino et al. 2020; Carpita et al. 2020a).

5. Limitations and conclusion

This study should be considered in light of several limitations. Firstly, it was conducted in a small sample, thus limiting the impact and the extensibility of our results. Moreover, significant differences in sex and age were reported among groups, and this may have led to biases also with respect to the possible changes in biochemical parameters related to these variables. In addition, several subjects, in particular within the ASD group, were affected by other disorders in comorbidity and/or were under pharmacological treatment, and while we have performed further analyses in order to assess differences depending on the presence of comorbidity/specific treatments, this sample characteristic may still have affected our results. Another limitation that should be considered is the lack of information on the nutritional state of the subjects, which may also have influenced our data. Furthermore, we used, excluding the SCID-5, self-reported psychometric instruments. While we employed instruments considered reliable in the literature, this specific feature should be considered closely, as subjects might have over/underestimated their own symptoms, leading to biases in the results. Lastly, the cross-sectional design of the study prevented us from hypothesizing possible temporal or causative relationships between the analyzed variables. Despite these limitations, our results seem to confirm, also in adult populations, the role in ASD of an unbalanced TRP metabolism, with lower TRP and KYNA concentrations. These alterations may eventually be associated with an imbalance between the neuroprotective/neurotoxic branches of KYN routes and with altered glutamatergic activity (Bryn et al. 2017). In this framework, our findings suggest, in agreement with previous studies, to reconsider the importance of hyperserotonemia as a mark of ASD, in particular in adult subjects (Hranilovic et al. 2007; 2009), which may instead feature lower levels of 5-HT (in particular in the PPP), as reported also by previous findings and in line with the beneficial effects of the SSRIs in this population (Spivak et al. 2004; Hollander et al. 2012; Harrington et al. 2013). On the other hand, further research should broadly investigate the alteration of TRP metabolism in ASD, considering all its pathways and branches. Moreover, as suggested by the altered IL-6 and HCY levels reported herein, the present study may confirm the involvement of immune system activation and of HCY-related metabolism impairment in autism spectrum, stressing also a possible statistically predictive role of these alterations for the presence of ASD

or even BAP (Melnyk et al. 2012; Han et al. 2015; Carpita et al. 2020a). Altered HCY metabolism might be associated with altered methylation processes and increased oxidative stress states, other factors which were hypothesized to be involved in the autism spectrum pathophysiology (Melnyk et al. 2012; Han et al. 2015). Correlations between psychometric scales and biochemical parameters also suggested different patterns of association between 5-HT (PPP), TRP, KYNA, HCY and IL-6 alterations and specific autistic dimensions (such as communication impairment, altered response to sensorial stimuli, inflexibility traits, or ruminative thinking) that in some cases support previous findings, and should be confirmed by further investigations (Harrington et al. 2013; Moustafa et al. 2014; Eszlari et al. 2016; Lehmann et al. 2016; Vidal et al. 2020; Karthik et al. 2020). As previously reported, the pathophysiology of autistic symptoms may feature a crucial involvement of impaired 5-HT and KYNA pathways, as well as of HCY-related metabolism and immune system alterations, eventually with intertwined interactions and different impacts on specific clusters of symptoms (Luchowska et al. 2005; Han et al. 2015; Bryn et al. 2017; Savino et al. 2020; Carpita et al. 2020a). Finally, the present study further supports the presence of a BAP among relatives of ASD probands, highlighting a continuum between sub-threshold and full-threshold ASD phenotypes not only from a psychopathological point of view. The intermediate alterations of biochemical variables in the BAP group reported in this study confirms, in adults, data from the limited number of previous biochemical investigations focused on this specific population, which were mainly led on children and their siblings or compared children with adult parents. In line with neuroimaging studies, which highlighted the presence of intermediate alteration in ASD relatives, these results stress the importance in ASD studies and in psychiatry of a dimensional approach, which should not be limited to psychopathology but also include somatic correlates (Billeci et al. 2016; Dell'Osso et al. 2019a). The specific association of functional impairment with increased HCY and IL-6 levels may eventually suggest a mirroring between higher inflammatory/oxidative state, impaired methylation processes, and loss of function (Martin and Grotewiel 2006; Sartori et al. 2012). HCY and IL-6 were also the parameters more associated with ruminative thinking, eventually confirming the involvement of ruminative thinking in worsening the impact of psychopathology (Nolen-Hoeksema et al. 2008; Dell'Osso et al. 2019a; 2019c; 2020b). Correlation between biochemical parameters, reporting negative correlations between

KYNA and HCY, may suggest, as previously hypothesized, possible interactions between these metabolic routes, with potentiating or counterbalancing effects (Luchowska et al. 2005; Savino et al. 2020). Further research investigating biochemical correlates should proceed in the framework of the possible presence of intertwined relationships between different systems and metabolic routes, as well between central and peripheral systems in shaping both neurodevelopment and psychopathological trajectories (Dell'Osso et al. 2019a; Carpita et al. 2020a). Our results also suggest the utility of metabolomics/proteomics approaches in this field of research, which could take into account multiple variables and metabolic signatures (Abraham et al. 2019; Glinton and Elsea 2019). Broadening the investigation in this field, while increasing knowledge on biochemical correlates of autism spectrum, may lead to improvements in diagnostic procedures and eventually to the identification of possible new treatment targets for this population. Moreover, it may lead to a better understanding of the multiple relationships between different systems and metabolic routes involved in CNS development and pathophysiology. As reported above, the cross-sectional design of our study prevented us from evaluating temporal relationships between the variables and, on the other hand, in the literature the possible consideration of psychiatric symptoms as a consequence or a cause of impaired peripheral systems is still debated (Eisenberg et al. 2017; Carpita et al. 2020a). However, as previously hypothesized by other authors, the relationship between ASD and altered biochemical processes might be considered bi-directional: while metabolic route or immune system alterations evaluated here may be implied in the behavioral, emotional and cognitive impairment linked to ASD, it is also possible that impaired cognitive and emotional processes may affect other systems besides CNS, as in the case of the hypothesized role of ruminative thinking in increasing pro-inflammatory activity (Eisenberg et al. 2017). Further studies in wider samples and possibly with a longitudinal design are warranted in order to clarify the relationship between the investigated biochemical systems and adult autism spectrum.

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