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Targeting the inherited basis of ASD for diagnosis and treatment by identification of molecular predictive profile

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Introduction

Autism Spectrum Disorders (ASD): Clinical features and Prevalence

Autism Spectrum Disorders (ASD) are a heterogeneous set of neurodevelopmental disorders characterized by deficits in communication and social interaction as well as the presence of restricted interests and repetitive behaviors, according to the Diagnostic and Statistical Manual of Mental Disorders, fifth edition (DSM-5)[1]. By being a spectrum, this condition is also characterized by a high degree of heterogeneity in the phenotypic manifestations, wide variation in levels of intellectual and language development, intra-individual differences in cognitive profiles, and frequently reported comorbidity with other psychiatric and developmental disorders[2]. Due to the unique deficiencies and high comorbidity, ASD is one of the most debilitating developmental illnesses and has a significant financial impact[2]. The term "autism" was coined in 1908 by Swiss psychiatrist Eugen Bleuler to describe withdrawal from reality in patients with schizophrenia[3]. Subsequently, in 1943, Leo Kanner directed this term towards a group of children without schizophrenia but exhibiting social isolation and language disorders. One year later, Hans Asperger identified children with social isolation but without the typical language anomalies of autistic children, diagnosing a new autistic-like disorder known as Asperger's Syndrome. ASD, Asperger's Syndrome (AS), Pervasive Developmental Disorder Not Otherwise Specified (PDD-NOS), Rett Syndrome, and Childhood Disintegrative Disorder (Heller's Syndrome) are five pervasive developmental disorders (PDD) included in the fourth edition of the DSM by the American Psychiatric Association (APA)[4]. Children with PDD exhibit different disorders falling into three specific areas: social interaction, communication, and restricted and repetitive behaviors[3]. Since each disorder has a wide variation in the severity of symptoms that tend to overlap across different groups, the DSM-5 has grouped PDD disorders into the broader and well-known category of ASD. To confirm the diagnosis of ASD, patients must demonstrate at least three symptoms of social communication and at least two symptoms in restricted and repetitive behaviors.

Despite advancements, reliable biomarkers for ASD currently do not exist. The current clinical diagnosis of ASD follows the criteria outlined in the DSM-5 by APA. In this framework, the clinical features associated with the condition fall within the triad: language, social interaction, and restricted or repetitive behaviors[1]. Among the various diagnostic tools available to confirm ASD in patients, the Autism Diagnostic Observation Schedule-II edition

(ADOS-2) and the Autism Diagnostic Interview, Revised (ADI-R) are widely recognized. Both adhere to the diagnostic criteria outlined in the DSM-5[5]. ADOS is considered the gold standard, representing the most accurate diagnostic test for confirming ASD. Specialists use this tool to evaluate individuals suspected of autism, ranging from non-verbal children to adults without verbalization disorders. ADOS comprises four modules tailored to the chronological age and linguistic development of the individual with suspected ASD. Diagnosis is established through the observation of social behaviors and communication. On the other hand, ADI-R involves an interview with parents and caregivers of children and adults suspected of autism, with a mental age exceeding 2 years. The combination of ADOS and ADI-R allows for a formal diagnosis, referencing the complete developmental history of the individual. This comprehensive assessment aids in determining the severity of the condition and facilitates the planning of therapy or an educational program based on the collected information[5,6].

Numerous disorders can coexist with ASD, encompassing both psychological and physiological symptoms and comorbidities. Psychological comorbidities include Attention Deficit Hyperactivity Disorder (ADHD), anxiety, depression, intellectual disability, Obsessive-Compulsive Disorder (OCD), impulsivity, and seizures. There are many neurological similarities between ASD and ADHD; indeed, hyperactivity and social deficits are symptoms present in approximately 40% of individuals diagnosed with ASD. Individuals with ASD often face communication difficulties and social isolation, leading to conditions of anxiety and depression. Other observed abnormalities include aggression and self-harm. Among common physiological comorbidities are gastrointestinal disorders, epilepsy, and sleep disorders[7]. Risk factors such as prenatal environment and synaptic defects contribute to a higher likelihood of developing epilepsy in individuals with ASD and intellectual disability. The immune system plays a significant role in ASD, with immune dysfunction recognized as a comorbidity in individuals with ASD exhibiting increased cytokine levels and neuroinflammation. Maternal immune activation appears to be a risk factor for ASD. Understanding associated conditions and providing accurate diagnoses can guide appropriate therapeutic interventions to improve the quality of life for these patients. Since ASD encompasses multiple comorbidities and clinical disorders, it results in a severe and debilitating condition for which, as of today, there are no diagnostic biomarkers and targeted medications.

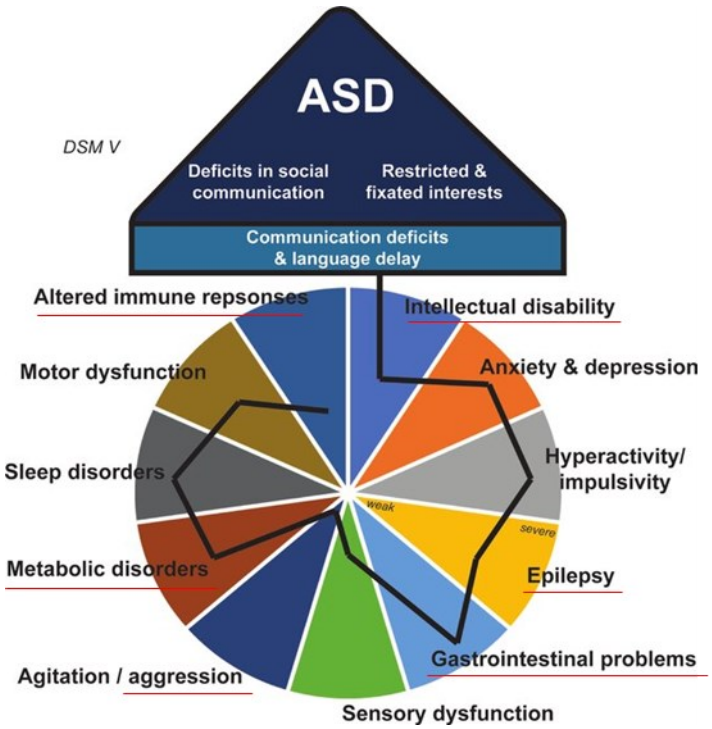


Figure 1. Clinical features of ASD. The three core features of ASD are deficits in social communication, restricted and fixated interests, and speech deficits and language delays. The first two features are used to diagnose ASD according to the DSM-5. In addition, individuals with ASD may present further symptoms and comorbidities such as cognitive deficits (intellectual disability), anxiety, depression, attention deficits and hyperactivity, impulsivity, seizures, gastrointestinal problems, sensory dysfunction, aggression, metabolic disorders, sleep disorders, motor dysfunction, and altered immune responses. However, not all symptoms and comorbidities are present in individuals with ASD, and those that are, vary in severity. This results in a considerable heterogeneity of clinical features of individuals with ASD. Figure adapted from Sauer et al. 2021[2] with modifications highlighting some of the comorbidities considered in the present study.

The most recent prevalence estimates worldwide are rather inconsistent with each other. The systematic review by Zeidan et al.[8] found that data published from 2012 to 2021 from 34 countries reported a median prevalence of 100 per 10,000 children; whereas the meta-analysis by Salari et al. found that several studies published from 2008 to 2021 reported a common prevalence of 60 per 10,000 children[9]. The three-level meta-analysis by Talantseva et al. revealed that the prevalence of ASD is significantly higher in North America (1.01%) than in Europe (0.73%) and Asia (0.41%), with the highest prevalence in the United States (1.12%) and the lowest in Taiwan (0.11%)[10]. Salari et al. found similar results where the prevalence was 1% in America and 0.4% in Asia, while in Europe the frequency was 0.5%. The most significant estimate of ASD prevalence was from Australia, at 1.7%[9].

Meta-analysis studies have revealed that the male-to-female ratio was 4.3 for ASD and 4.2 for AD[8,10]. A more selective systematic review has reduced this ratio to 3.32[10]. In terms of age, children aged between 6 and 12 years have shown a significantly higher prevalence of ASD (0.82%) compared to children under 5 years (0.60%) and children over 13 years (0.57%). Younger ASD children may not be identified until entering primary school or later, where social demands increase, and deficits become more apparent[10]. According to the Autism and Developmental Disabilities Monitoring Network (ADDM) of the Centers for Disease Control and Prevention (CDC), several studies on ASD prevalence rates have been conducted in the United States from 2000 to 2018[11]. The prevalence of ASD among 8-year-old children was 67 per 10,000 in 2000, while in 2018, ASD affected 230 children per 10,000[12]. The reported frequency of ASD in Europe varies widely based on demographic context and region. Germany, France, Belgium, Ireland, and Italy reported prevalence rates of 0.4%, 0.4%, 0.6%, 1.1%, and 1.15%, respectively[13–17]. As previously indicated by published systematic reviews, the observed differences in prevalence estimates over time are likely not attributable to a genuine increase in prevalence. Instead, these variations are associated with changes and enhancements in diagnostic categories, research methodology, and overall research quality. Other contributing factors include increased accessibility to diagnostic and intervention services, heightened awareness of ASD within both professional and non-professional communities, and recognition that ASD can coexist with other disorders[18,19]. Beyond historical trends, there are additional sources of variation, including geographical location, national income, the applied design of research studies, utilized diagnostic criteria, the age of the studied population, and other socio-demographic characteristics[8,9,20].

ASD Etiology: Complex Interplay of Genetic, Epigenetic, and Environmental Factors

Given the broad inter- and intra-individual heterogeneity of ASD, the etiology is likely multifactorial. A complex integration of various genetic, epigenetic, and environmental factors plays a significant role in the pathogenesis of ASD[21]. Various risk factors have been explored in an attempt to understand the causes of this wide spectrum of disorders. Early studies on homozygous ASD children suggested high heritability, but the results obtained did not identify the causes of ASD[22]. Consequently, researchers expanded their studies in an attempt to find a connection between genetic and epigenetic components and parental and environmental influences that may predispose to ASD[23]. Initially, ASD etiology research focused on individual genes responsible for autism. However, only 1-2% of individuals with ASD have specific genetic syndromes, such as Rett syndrome associated with the mutation in the *MECP2* gene (Xq28), X-fragile syndrome associated with the *FMR1* gene (Xq27.3), and tuberous sclerosis associated with the *TSC1* and *TSC2* genes (9q34.13 and 16p13.3, respectively)[24]. These cases represent only a small part of ASD individuals; in fact, idiopathic ASD represents the majority of cases, where over 400 genes have been correlated[25]. Examples of genes associated with idiopathic ASD are the *NLGN3* and *NLGN4X* genes encoding adhesion proteins on the postsynaptic membrane that mediate the formation and maintenance of synapses between neurons[26]. Another gene involved in the assembly of postsynaptic structures, *SHANK3* gene, has been associated with cases of ASD with severe verbal and social deficits[26]. The numerous genes identified as risk factors for ASD are genes that function in chromatin remodelling, transcriptional regulation, or synaptic architecture and functionality[25,27]. Not all children with a predisposing genotype develop ASD, suggesting that genetic factors alone cannot explain the causes of ASD. To these, the role of environmental factors interacting with the genome through epigenetic mechanisms influencing gene expression and neurotypical development needs to be further explored.

It is believed that the gene-environment interaction determines different ASD phenotypes for each patient. Whole human genome studies have identified other genetic factors predisposing to ASD, such as copy number variants (CNV)[28]. In particular, CNVs in the *PARK2* gene, which plays a role in neurological development and is associated with Parkinson's disease and early-onset schizophrenia[29,30]. Another line of research has identified a significant association with ASD of 16 SNPs in the oxytocin receptor gene, *OXTR* gene, implicated in behaviour and social bonding[31].

As known, individual genes or chromosomal changes cannot alone explain the etiology of ASD. Therefore, another possible scientific avenue is epigenetic influence. Epigenetic changes, such as DNA methylation and histone modifications, influence gene activity and expression without altering the gene sequence and can reflect environmental influences on genes[32]. Individuals with ASD have been identified with higher levels of methylation in the promoter region of the *OXTR* gene and, consequently, reduced gene expression. Other candidate genes in ASD influenced by DNA methylation are *BCL2*, *RORA* , *MeCP2*, *OXTR*, and *SHANK3* genes[33–37]. The combination of whole-genome DNA methylation data with transcriptome data in ASD shows that genes associated with immune regulation are hypomethylated and overexpressed, while genes related to synaptic function are hypermethylated and underexpressed[38].

Recent studies have identified some environmental factors influencing susceptibility to ASD, including the nutritional and metabolic status of the mother, parental age, infection during pregnancy, prenatal stress, and exposure to toxins, drugs, or heavy metals[23]. Environmental factors can induce epigenome disorders during critical periods of neurological development, affecting cells during proliferation, migration, and terminal differentiation processes[38]. At the molecular level, these disorders include changes in DNA methylation, histone modifications, and the expression of various non-coding RNAs. During gestation, the mother's lifestyle can expose the fetus to a wide range of environmental insults. Maternal nutritional deficiencies or excesses, such as folic acid, zinc, iron, vitamin D, and omega-3, can directly influence fetal neurological development through epigenetic mechanisms[39]. In fact, since methylation is strictly interconnected with the available methyl groups donated by S-adenosyl-methionine (SAM) to DNA methyltransferases (DNMTs), mediating epigenetic modification at specific DNA sites[40], folate, being one of the main sources of methyl groups donated to SAM through the one-carbon metabolism pathway[41], is essential for successful pregnancy. Which is why, during pregnancy, mothers need to take vitamin supplements, including the B-vitamin family[42]. Folic acid plays a crucial role in preventing neural tube defects and ensures an adequate supply of methyl donors before and during gestation[43]. Case-control studies have observed a higher risk of ASD in children whose mothers did not assimilate sufficient amounts of folic acid or who had defects in genes associated with one-carbon metabolism, such as the *MTHFR* gene[44,45]. ASD has also been associated with immune system dysfunction important during neurological development[46]. Maternal infection is another non-genetic factor that increases the risk of ASD[47,48], as

infections during pregnancy can alter the maternal immune system, causing inflammation and excessive cytokine production in the fetus, resulting in genetic dysregulation[49,50]. Indeed, abnormal adaptive and innate immune function has been observed in individuals with ASD[51]. Elevated levels of chemokines and cytokines in the central nervous system and peripheral blood, along with glial cell activation, determine a neuroinflammatory phenotype observed in ASD[52]. Immune risk factors such as maternal infection and immune activation during pregnancy, immune dysregulation, inflammation, and microbial dysbiosis can influence the pathogenesis of ASD during prenatal and postnatal periods[53]. It has been recognized that the gut-microbiome-brain axis modifies behaviour and influences neurological development[2,54]. Dysbiosis of the gut microbiome alters intestinal permeability, causing inflammatory processes that affect the nervous system and contribute to ASD neuropathology[54,55]. Given the numerous described evidences and the multiple factors that potentially can determine ASD phenotypes, a multidisciplinary approach is necessary to identify biomarkers and treatment strategies for applying personalized medicine to the heterogeneity of ASD individuals.

Cytokine Dysregulation in ASD

In the effort to understand the causes of ASD, increasingly compelling evidence indicates that cytokine dysregulation might be involved in the pathogenesis of ASD, and cytokine levels could potentially serve as biomarkers to define subgroups of this disorder[56]. In the immune system, cytokines constitute a heterogeneous group of proteins that act as immunomodulatory and endocrine messengers involved in the regulation of inflammation and neurological development[57]. Altered levels of cytokines reflect immune dysregulation and have been associated with ASD patients. Male children with high-functioning ASD exhibit elevated levels of IL-1β, IL-1RA, IL-5, IL-8, IL-12(p70), IL-13, and IL-17 compared to matched controls[58]. IL-1β, a proinflammatory cytokine expressed early in the immune response, activates neutrophils and macrophages to eliminate invasive pathogens, thereby propagating tissue-level inflammation and inducing the production of IL-6 systemically[59]. Children with ASD also produce excessive amounts of the IL-1 antagonist, IL-1RA, which functions to reduce inflammation by competing with the IL-1β receptor, and increased levels of which may represent an attempt to counteract inflammation in ASD[58]. IL-1β is involved in the production of IL-17, which, in turn, stimulates the production of IL-8, a crucial chemokine in the innate immune system. Compared to individuals with typical development, ASD patients also show increased expression of IL-1β, TNF-α, IL-6, IL-12, and IL-23, suggesting dysregulated immune response[60]. TNF-α is considered a central regulator of inflammation and has been found elevated in cerebrospinal fluid in subjects with ASD[61]. IL-6 is a proinflammatory cytokine produced during the acute phase of the immune response, is involved in the development and functioning of the nervous system and can alter synaptic networks[62]. Furthermore, TNF-α, IL-6 and IL-1β are neurocytokines considered molecular signals of disease because they are able to cross the blood-brain barrier and act on the hypothalamus where they promote fever and sick behaviour[63,64].

Studies highlight that aberrations in cytokine production may contribute to the pathogenesis of ASD and represent a significant factor in explaining differences in clinical phenotypes and comorbidities that influence the severity and course of ASD[56]. Studies conducted on cerebrospinal fluid and peripheral blood demonstrate a link between immune dysfunction and behavioural traits on the disorder spectrum[65]. The role of adaptive immunity in neural development disorders such as ASD is supported by alterations found in subsets of T and B cells and antibody levels in blood, cerebrospinal fluid, and brain tissues[56,66] (figure 2). ASD subjects present an increase and decrease in the pro-inflammatory response of T helper-2

and T helper-1 cells, respectively[67]. Studies on the possible correlation of the immune system to ASD have also reported an imbalance between helper-suppressor T cells and regulatory T cells[56]. ASD children with dysregulated T cell activity showed excessive production of cytokines such as IL-5, IL-12, IL-13, IL-17, IL-21, IL-22, and IL-23[68]. Cytokines produced by lymphocytes impact brain development; for example, IL-17A produced by Th17 CD4+ T cells is associated with maternal immune activation, is upregulated in autoimmune and neurological diseases, and is positively correlated with ASD severity[69–71]. ASD individuals exhibit a proinflammatory cytokine profile associated with severity, social and behavioural deficits, hyperactivity, and typical irritability of the disorder[56]. Stereotyped behaviour in autism seems to be correlated with elevated concentrations of IL-1β, IL-6, IL-8, IL-12p40, TNF-α and IFN-γ, and low concentrations of TGF-β1 and GM-CSF[72]. Reduced levels of TGF-β1 have been associated with reduced adaptive behaviour and worsening behavioural symptoms[73,74]. TGF-β and IL-10 are down regulated in association with hyperactivity in ASD individuals who upregulate IL-1β, IL-6, IL-8, and IL-12(p40)[72,73,75]. Meanwhile, aggressive behaviour in ASD children has been correlated with increased expressions of IL-1β, IL-6, IL-10, and MCP-1[76]. Studies correlating cytokines with ASD severity have observed that more severe patients show higher levels of IL-1β, IL-6, IL-12p70, TNF-α and IL-17A[56]. Typical social disturbances in autism have been correlated with upregulation of cytokines such as IL-1β, IL-6, IL-10, MCP-1, MIP-1β, MIP-1δ, GM-CSF, and MIF, and downregulation of IL-23, TGF-β1, and TNF-α[56,76]. All these pieces of evidence suggest that cytokines could be considered potential biomarkers for subgroups of individuals diagnosed with ASD.

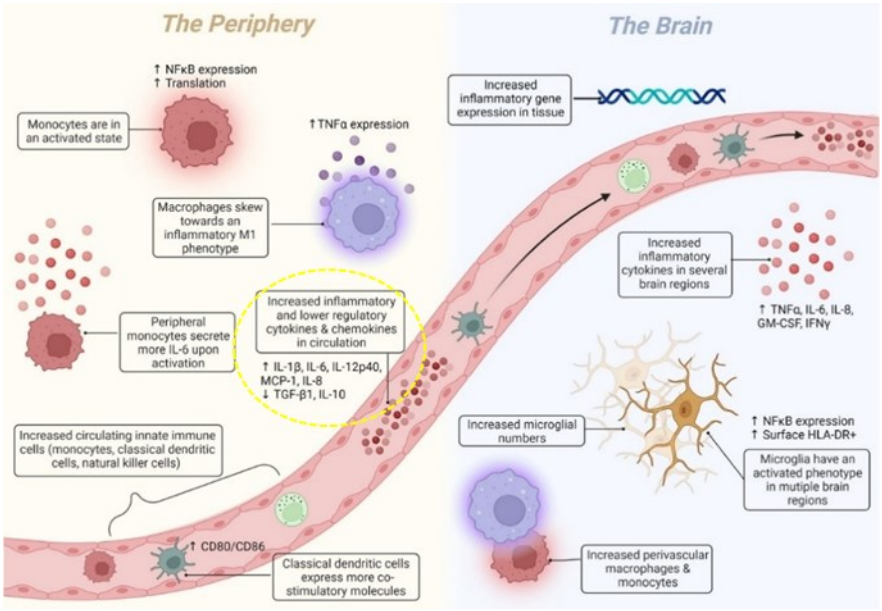


Figure 2. Cellular and cytokine dysregulation of the innate immune system in both the periphery and brain of individuals with ASD. Inflammatory cytokines produced mainly by cells of the innate immune system are elevated in the periphery of ASD individuals. Increased frequencies and altered activation of innate cells such as monocytes, macrophages, and dendritic cells are found, contributing to the establishment and perpetuation of inflammation in ASD. Notably, inflammatory cells and cytokines of the innate immune system can have consequences on brain homeostasis. Peripheral cells and cytokines can breach barriers in the brain and upregulate inflammatory gene expression. Inflammatory conditions in the brain may also alter tissue-resident immune cells, as altered microglial activation and cellular density have been documented in several regions of the ASD brain. CD80, cluster of differentiation 80; CD86, cluster of differentiation 86; GM-CSF, granulocyte macrophage colony-stimulating factor; HLA-DR, human leukocyte antigen-DR isotype; IFNγ, interferon-gamma; IL-1β, interleukin-1beta; IL-6, interleukine-6; IL-8, interleukin-8; IL-10, interleukine-10; IL-12p40, interleukin-12subunit40; MCP-1, monocyte chemoattractant protein-1; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; TGF-β, transforming growth factor-beta; TNFα, tumor necrosis factor-alpha. Figure adapted from Huges *et al.* 2023 with modifications highlighting the variations in circulating cytokines [77].

Transgenerational DNA Methylation and Gut Microbiota in ASD

The pathology of ASD is complex and involves numerous factors, including genetic, epigenetic, and environmental factors. Genetic factors account for only a small percentage of ASD cases, indicating that the onset of the disorder requires additional environmental influences[78]. Recent studies have highlighted the involvement of the gut microbiota in increasing the risk of ASD. It is hypothesized that environmental exposure can alter the parental microbiota, subsequently increasing the risk of ASD in offspring[79,80]. It is well known that in ASD, the alteration of central nervous system development and thus the impairment of normal brain functions leads to the onset of the disorder, but how this occurs remains unknown. Further studies have identified the interaction between the central nervous system and the gut microbiota through the gut-brain axis[54]. This axis is involved in the pathogenesis of ASD, but the underlying mechanisms remain elusive[54].

In this broad disorder, epigenetic factors such as DNA methylation play a crucial role. Alteration of DNA sites subjected to epigenetic modifications has been associated with ASD[81]. The gut microbiota can influence the methylation status of epithelial cells in diseases such as colorectal cancer[82]. Furthermore, several studies suggest that these epigenetic modifications can be inherited and exert a regulatory function in the development of offspring[83,84].

Some studies have focused on the interaction between the gut microbiota and DNA methylation in ASD. One study highlighted the interaction between the gut microbiota of autistic children and their mothers by examining mother-child pairs of ASD children and pairs of healthy children[85]. Additionally, animal studies have shown that transplanting the gut microbiota from ASD subjects into antibiotic-treated mice led to colonization by the ASD microbiota, and these mice exhibited ASD-like behaviours[86]. To demonstrate the involvement of DNA methylation and its transgenerational transmission, researchers analysed the epigenetic modifications inherited by the offspring of mice transplanted with the gut microbiota of ASD subjects[87]. This study demonstrated that the gut microbiota transplantation induced changes in the methylation levels of genes associated with ASD in the parental mice, and some of these sites were transmitted to the offspring, which exhibited typical ASD behaviours. These findings highlight an inherited epigenetic component in ASD subjects from asymptomatic parents[87]. In light of these discoveries, it is of interest to further

investigate the transgenerational transmission of DNA methylation between asymptomatic parents and ASD offspring.

One-Carbon Metabolism Pathway in ASD

It is now established that ASD is a multifactorial condition involving both genetic factors and a wide range of environmental factors, and only recently has research on environmental risk factors significantly increased[88]. Initially, it was believed that the environmental contribution was negligible due to the limited understanding of gene-environment interactions[89]. Currently, studies have focused on critical time periods for neurological development, identifying potential environmental risk and protective factors in ASD, such as diet and nutritional status of children with ASD, to investigate the direct association between maternal nutrition and the risk of ASD in offspring[88,89]. As is well-known, maternal nutrition is essential for the fetal brain's development, and a deficiency of essential nutrients in the diet significantly increases the risk of adverse outcomes in neurological development, such as neural tube defects and schizophrenia[90]. Maternal nutritional balance is indispensable for the proper structural and functional development of the fetal brain, and therefore, a deprivation of essential nutrients during pregnancy can influence the risk of ASD[91].

Various environmental factors can contribute to the development of ASD. Advanced maternal age (35 years and older), maternal chronic hypertension, preeclampsia, gestational hypertension, and overweight before or during pregnancy are just a few examples[92]. Among the risk factors for ASD, metabolic disturbances are found. Several studies suggest that ASD is associated with altered energy metabolism, increased oxidative stress, decreased methylation capacity, and altered sulfur metabolism[93–95]. Particular attention is paid to the folate cycle, as disruptions in the folate-methionine cycle have been highlighted in many individuals with ASD[96–98], suggesting that this cycle may play an essential role in the pathogenesis of autism[41]. Therefore, the various players and metabolites associated with this cycle could be used as potential biomarkers and therapeutic targets for ASD.

Folate, an essential substrate in the folate cycle, plays a key role in numerous vital processes in mammals. These include DNA synthesis and repair, purine and pyrimidine synthesis, amino acid synthesis, as well as histone and DNA methylation. Methylation of histones and

DNA is one of the major epigenetic processes that influence gene expression in both health and disease conditions[99–101].

Folate, also known as folic acid or Vitamin B9, is a water-soluble vitamin considered essential for pregnant women. Since this vitamin cannot be synthesized by the human body, it must be obtained through the diet or via supplements[43]. Unlike folate, folic acid is not naturally present in foods but is produced synthetically for use in food fortification and as a dietary supplement. Folate is found in various natural food sources, such as leafy green vegetables, citrus fruits, beans, peas, lentils, seeds and nuts[102]. It is also present in some animal-derived foods like liver and kidneys but can be destroyed during the cooking process[103]. During pregnancy, the requirement for folate increases and becomes crucial for both mother's health and fetal development. A daily supplement of folic acid (400 mcg/day) should be taken during the first trimester of pregnancy[104], which helps prevent the occurrence of congenital neural tube defects, affecting the embryonic nervous system, which could compromise the growth and development of the fetus[102,105].

The absorption of folate in the intestinal lumen by enteric cells and in other tissues such as the kidney, liver, placenta, and choroid plexus is mediated by two folate transporters: the proton-coupled folate transporter (PCFT) and the reduced folate carrier (RFC)[106] (figure 3, step 1). When folate is ingested, it passes from enteric cells to the hepatic portal vein through the basolateral membrane to reach the liver. In hepatic cells, folate is absorbed by PCFT and RFC and can be stored in hepatocytes, secreted into bile, or released into the bloodstream. Through the bloodstream, folate is transported to various tissues, and its absorption is regulated by three glycoproteins known as folate receptors alpha (FRα), beta (FRβ), and gamma (FRγ)[107]. The predominant circulating form of folate in the blood is 5-methyltetrahydrofolate (5-methyl-THF), which has a higher affinity for FRα than for FRβ and FRγ. 5-methyl-THF is one of the major methyl group donors involved in the conversion of homocysteine (Hcy) into methionine[108].

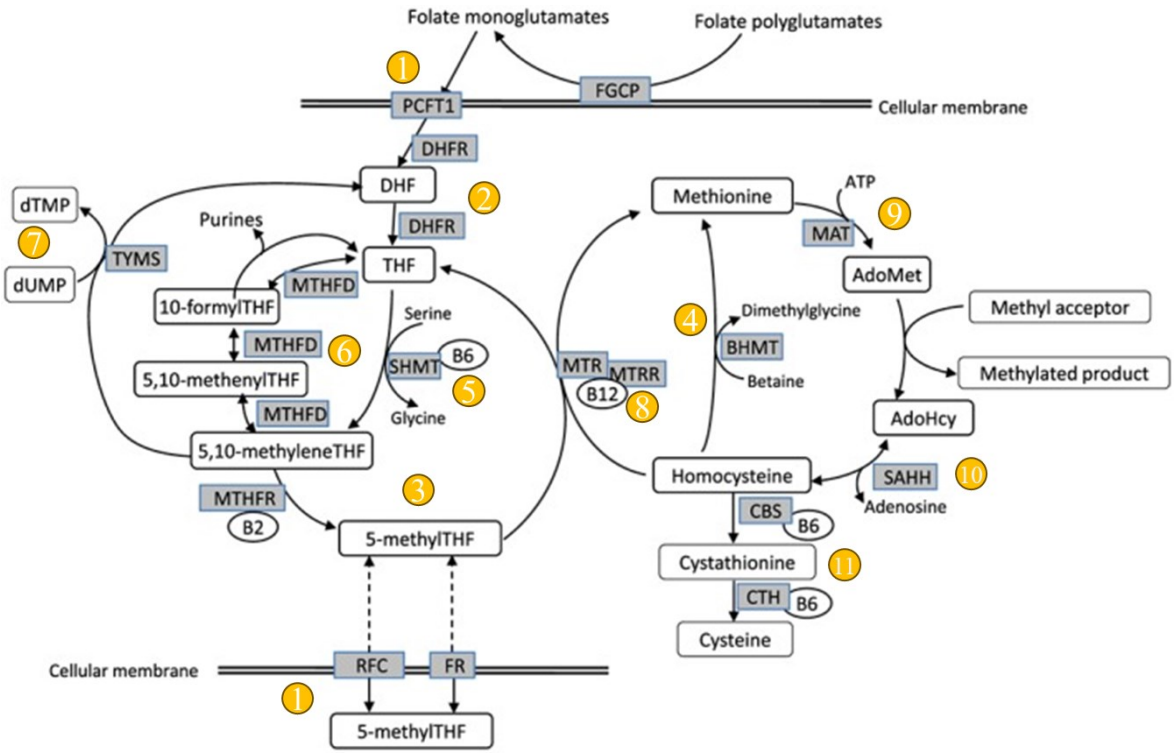


Figure 3. One-Carbon Metabolism Pathway: Overview of the folate cycle, remethylation cycle, and transsulfuration pathway. Complex network of biochemical reactions essential for various cellular processes, including DNA synthesis, methylation reactions, and nucleotide biosynthesis. It involves the transfer of one-carbon units, typically in the form of methyl groups, between different molecules. This pathway plays a critical role in maintaining cellular homeostasis and is closely linked to the regulation of gene expression, cell growth, and development. AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; BHMT, betaine-homocysteine methyltransferase; CBS, cystathionine β-synthase; CTH, γ-cystathionase; DHF, dihydrofolate; FGCP, folyl poly-γ-glutamate carboxypeptidase; FR, folate receptor; MAT, methionine adenosyltransferase; MTHFD, methylenetetrahydrofolate dehydrogenase; MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; MTRR, methionine synthase reductase; PCFT1, proton-coupled folate transporter; RFC, reduced folate carrier; SAHH, S-adenosylhomocysteine hydrolase; SHMT, serine hydroxymethyltransferase; THF, tetrahydrofolate; B2, Vitamin B2; B6, Vitamin B6; B12, Vitamin B12. Figure adapted from Hiraoka et Kagawa 2017 with modifications highlighting (yellow labels) the main steps of the pathway.[108].

Dietary folates primarily exist as polyglutamates, which need to be deconjugated into monoglutamates before absorption, that is, before they are transported into enteric cells. In contrast, folic acid has a completely oxidized pteridine ring conjugated to a single glutamate residue[109]. Folate monoglutamates, including folic acid, require further processing to enter folate metabolism, as they need to be first reduced to dihydrofolate (DHF) and then to tetrahydrofolate (THF) by the dihydrofolate reductase (DHFR) enzyme[110] (figure 3, step 2). Folate molecules serve as carriers for one-carbon (1C) units, allowing them to be

manipulated and assembled to support metabolic processes. Within the folate cycle, 1C units bound to THF can be interconverted between different oxidation states, with 5,10-methylene-THF, 5-methyl-THF, and 10-formyl-THF each supporting distinct biosynthetic functions[111]. Another formyl-THF species, 5-formyl-THF, does not play a direct biosynthetic role but serves as a 1C reserve. The most reduced form of folate 1C unit, 5-methyl-THF, has a unique cellular fate, which is the remethylation of homocysteine to form methionine[112]. 5-methyl-THF is produced by the NADPH-dependent cytosolic activity of methylene tetrahydrofolate reductase (MTHFR) and is consumed by the cobalamin (Vitamin B12)-dependent enzyme methionine synthase (MTR)[96] (figure 3, step 3). Mammalian cells can also regenerate methionine from homocysteine independently of folates using betaine or trimethylglycine, a product of choline degradation, through the betaine-homocysteine methyltransferase (BHMT) enzyme (figure 3, step 4). BHMT expression is limited to the liver and kidneys[113], whereas widespread expression of MTR allows homocysteine remethylation throughout the body.

THF can be directly converted into 5,10-methylene-THF by the vitamin B6-dependent enzyme serine hydroxymethyltransferase (SHMT) (figure 3, step 5). This reversible reaction simultaneously converts glycine into serine. Cells can use SHMT to produce serine in one compartment and catabolize it in another, with the direction of flow depending on the supply and demand of 1C units within each compartment[114]. The conversion of THF into 5,10-methylene-THF can alternatively proceed with the formation of two intermediates, 10-formyl-THF and 5,10-methenyl-THF, and is catalyzed by the trifunctional enzyme called methylene-THF dehydrogenase (MTHFD), which possesses formyl-THF synthase, methenyl-THF cyclohydrolase, and methenyl-THF dehydrogenase activities[115] (figure 3, step 6). 10-formyl-THF is the most oxidized form of folate carbon and can act as a one-carbon donor for purine synthesis, which is important for DNA and RNA synthesis[112]. 5,10-methylene-THF can have two different fates. In one case, 5,10-methylene-THF can donate a methylene group for the conversion of dUMP to dTMP, which is important for DNA synthesis (figure 3, step 7). This reaction is catalyzed by thymidylate synthase (TYMS) and produces DHF, which can be reconverted into THF by DHFR to re-enter the folate cycle[112]. The other fate of 5,10-methylene-THF involves its reduction by the riboflavin (Vitamin B2)-dependent enzyme MTHFR[116]. This enzyme is critical in regulating the availability of 5-methyl-THF for Hcy remethylation. The transfer of the methyl group from 5-methyl-THF to generate methionine is catalyzed by methionine synthetase (MTR), which uses cobalamin

(vitamin B12) as a cofactor, forming the Cbl(I)MTR complex (figure 3, step 8). When MTR binds the methyl group, the methylcbl(III)MTR complex is formed, which transforms back to Cbl(I)MTR when it adds the methyl group to Hcy to generate methionine and THF. After a series of reactions, the Cbl(I)MTR cofactor is oxidized, producing Cbl(II)MTR, which is unable to accept a methyl group from methyltetrahydrofolate and must be reactivated by reductive methylation; the one-electron reduction is catalyzed by methionine synthase reductase (MTRR), and the transfer of a methyl group from S-adenosylmethionine (AdoMet) regenerates the active methylcobalamin form of the enzyme[117](figure 3, step 8). The production of AdoMet from methionine and ATP is catalysed by methionine adenosyltransferase (MAT) (figure 3, step 9). The ultimate source of methyl groups for methylation processes, such as those involving proteins, RNA, DNA, and neurotransmitters, is AdoMet. Each of these processes creates S-adenosylhomocysteine (AdoHcy), an allosteric inhibitor of methylation. The enzyme S-adenosylhomocysteine hydrolase (SAHH) hydrolyzes adenosine and Hcy from AdoHcy (figure 3, step 10). AdoHcy production is favoured by the equilibrium of this reversible reaction, hence metabolizing Hcy and adenosine is necessary to keep AdoHcy levels low. Under the trans-sulfuration pathway, Hcy is converted irreversibly to cysteine by processes mediated by cystathionase γ-synthase (CTH)[108] and cystathionine β-synthase (CBS)[118], both of which need pyridoxal phosphate, or vitamin B6, as a cofactor (figure 3, step 11). The trans-sulfuration pathway is the only cysteine synthesis pathway in which the CBS enzyme catalyses the transfer of sulphur from Hcy to cysteine[118], a potent component of the antioxidant glutathione[119].

The transulfuration pathway, the methionine cycle and the folate cycle are the three main pathways of one-carbon metabolism crucial in epigenetic regulation during embryonic development[40].

High concentrations of methyl donors are required during neurogenesis[120], so folate plays an important modulatory role on the developing brain through DNA synthesis, neurotransmitters, and myelination. Therefore, numerous studies have been conducted to understand the possible causal relationship between folate intake, either before or during pregnancy, its metabolism, and the onset of ASD. Unfortunately, the results obtained are still mixed. A systemic review attempted to elucidate the association between folic acid and ASD by considering biological, genetic, and epigenetic evidence and it was found that maternal prenatal folic acid supplementation (600 μg) has a beneficial effect on the risk of onset of ASD disorders, but excessive nutritional enrichment can cause detrimental

circulation of unmetabolized folic acids in maternal blood. In ASD children, folic or folinic acid supplementation leads to an improvement in neurological and behavioural symptoms. Several authors report that MTHFR polymorphisms have a significant influence on ASD risk[116].

MTHFR: gene and function

Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme of folate metabolism in the process of one-carbon metabolism for the reaction of producing methyl groups to participate in epigenetic regulation[32]. The *MTHFR* gene is located on chromosome 1 (1p36.22), comprises 13 exons and encodes for a 697 amino acids (aa) protein product[121]. Specifically, MTHFR converts 5,10-methyleneTHF to 5-methylTHF by participating in folate conversion and the DNA methylation-related homocysteine remethylation cycle[122]. Defects and variants in the *MTHFR* gene result in reduced MTHFR enzymatic activity causing impaired methylation and folate deficiency. Numerous studies have been conducted on the link between MTHFR and human diseases, including cardiovascular diseases, cancers, neurological diseases, and psychiatric disorders[123–126]. As DNA methylation and folate are important for mental health, reduced MTHFR activity has been associated with the onset of several psychiatric diseases, schizophrenia, bipolar disorder, depression, autism, and attention-deficit/hyperactivity disorder[32,127]. It has been identified that the *MTHFR* gene has 14 single nucleotide polymorphisms (SNPs), common or rare, that are associated with enzyme deficiency[121]. Among these, the most studied SNPs that appear to be clinically significant are the C677T transition (rs1801133) and the A1298C transversion (rs1801131). These two variants can reduce MTHFR enzyme activity differently. Regarding C677T, the enzyme activity of heterozygous and homozygous mutant individuals is approximately 67% and 25% of that of wild-type individuals, respectively. In contrast, for A1298C, the enzyme activity of heterozygous and homozygous mutant individuals is about 83% and 61% compared to wild-type individuals, respectively[128]. It is estimated that more than 60% of the general population carries one of the two polymorphic alleles, and at least 10% of them carry both homozygous alleles (677TT or 1298CC) and/or compound heterozygotes, which are rarely in cis (CT/AC) but more commonly in trans (CT/CA) due to strong linkage disequilibrium[129].

The *MTHFR* C677T variant involves a change from cytosine to thymine at position 677 in exon 4, resulting in the substitution of alanine with valine (A223V), leading to a thermolabile enzyme with reduced enzymatic activity[130]. This condition is more pronounced under folate deficiency[129,131]. Specifically, the homozygous 677TT condition is associated with low folate levels and an increase in Hcy. During pregnancy, maternal folate status is determined by genetic background, and diet becomes important as it can influence susceptibility to ASD[132]. Several studies have demonstrated a higher frequency of the C677TT polymorphism in children with ASD compared to healthy controls[133–135]. Furthermore, a significant interaction between maternal genotype 677TT and an increased risk of ASD has been observed[44], suggesting that the *MTHFR* C677T polymorphism may be involved in the early development of ASD. A systematic review of eight meta-analyses has confirmed that the C677T polymorphism of the *MTHFR* gene is associated with an increased risk of ASD. This association is strong in allelic, dominant, and heterozygous models, while it is weaker in the recessive model. In conclusion, the *MTHFR* C677T polymorphism may contribute to the risk of ASD[136].

The second most studied variant of *MTHFR*, A1298C, causes a change from adenine to cytosine occurs at position 1298 in exon 7, leading to the substitution of glutamic acid with alanine (E429A). The result of this transversion is an enzyme with reduced activity, which is more detrimental in the homozygous 1298CC condition, although to a lesser extent compared to C677T[137,138]. The association of the A1298C variant with neural tube defects and mental disorders is controversial. One possible explanation for such varied results could depend on the combination of variants, genes, additional genetic polymorphisms, and nutritional factors[129,130]. Unlike the C677T polymorphism, the association between the A1298C variant and ASD is not significant when considering the overall population. However, excellent results are obtained when stratifying by ethnicity. In fact, stratification analysis has indicated that the *MTHFR* A1298C polymorphism might be a susceptibility factor for ASD in Caucasians. Nevertheless, further studies are needed to confirm this relationship, taking into account potential gene-gene and gene-environment interactions in ASD susceptibility[139]

The combined heterozygosity of the two *MTHFR* variants results in lower MTHFR enzyme activity compared to having each heterozygosity separately and leads to elevated Hcy levels and low folate levels, similar to individuals with the 677TT homozygous variant[140]. The cis haplotype *MTHFR* 677T/1298C is a rare condition and has been observed more frequently

in cases of spontaneous abortions compared to healthy newborns, suggesting strong adverse effects[141,142].

The role played by MTHFR in ASD is still relatively less studied compared to other mental disorders like depression and schizophrenia. Various reports highlight the potential role of the C677T and A1298C variants in ASD risk, as described in both preclinical [143]and clinical studies[32]. The role of *MTHFR* polymorphisms and folate status during pregnancy is relevant, as studies on mother-child dyads have shown a correlation between one-carbon metabolism gene genotypes and periconceptional vitamin intake in ASD[44]. Authors have pointed out higher frequencies of 677TT homozygotes in children with ASD compared to healthy controls, as well as a higher risk of having ASD children for mothers with the TT genotype who took fewer prenatal vitamins[44]. This suggests gene-environment interaction effects in the etiology of ASD, although they require further confirmatory studies along with exploration of potential interactions between susceptibility genes in other pathways, such as epigenetic ones involving methylation. Overall, it becomes clear that further genetics and epigenetics studies are needed, preferably focused on genome comparison in mother-child dyads.

DHFR: gene and function

The *DHFR* gene is located on chromosome 5 (5q14.1) and comprises six exons that encode a protein product of 187 amino acids[144,145]. The enzyme DHFR catalyzes the reduction of folate to DHF and subsequently to THF, utilizing NADPH as a cofactor[146]. DHF and THF are the two key isoforms for folate entry into the folate cycle related to Hcy metabolism, as well as in the de novo synthesis of a variety of essential metabolites, including amino acids, lipids, pyrimidines, and purines[147]. Different DHFR enzyme activity is crucial for the relative balance between optimal DNA methylation and faithful DNA replication[99]. Inhibition of DHFR reduces the availability of THF and consequently the production of pyrimidine and purines important for the synthesis of DNA and RNA. One of the major functional polymorphisms within the *DHFR* gene is a 19-base pair insertion/deletion (19bp ins/del; rs70991108) in the promoter/first intron of the gene[32]. The *DHFR* 19bp ins/del has been associated with alterations in folate levels in a comparison of healthy individuals. In particular, women with the 19bp del/del genotype had higher serum levels of the enzyme and red blood cell count compared to the ins/del and ins/ins genotypes, suggesting a reduced

risk of having children with neural tube defects[146]. In addition to playing a key role in folate metabolism, DHFR can regulate tetrahydrobiopterin (BH4), a crucial enzymatic cofactor required for the synthesis of serotonin, dopamine, and nitric oxide[148]. The net availability of BH4 reflects the balance between BH4 synthesis, BH4 loss due to oxidative stress, and BH4 resynthesis mediated by DHFR. Once produced, BH4 can act as an anti-inflammatory molecule and a scavenger of free radicals, protecting against oxidative stress. At the same time, BH4 is subject to auto-oxidation, leading to the release of superoxide radicals that contribute to inflammatory processes and the production of BH2, an inactive version of BH4, reducing its bioavailability. Changes in BH4 levels have been documented in a variety of pathological conditions, including Alzheimer's disease, Parkinson's disease, and depression, where they describe increased oxidative stress, inflammation, and alterations in monoaminergic function[149].

The *DHFR* 19bp ins/del has been associated with a significant risk of ASD due to its role in regulating folate status and the existing interaction between folate and the glutamatergic nervous system[150]. Folate isoforms are conjugated with at least one glutamate residue and can potentially have excitatory glutamate properties, competing with inhibitory transmitters for receptor binding, inhibiting glutamate decarboxylase activity and high-affinity glial glutamate uptake[151,152]. Glutamate pathways play a key role in neural plasticity and development, and the Autism Genome Project Consortium has identified loci associated with autism, such as the glutamate ionotropic receptor kainate type subunit 2 gene (GRIK2/GluR6), which is in linkage disequilibrium with autism[153,154]. Two polymorphisms within this gene act as a significant risk factor for this neurodevelopmental condition[153].
Synthetic folic acid is 100 times more potent than glutamate as an excitatory ligand in neuronal tissue. Fortunately, folic acid cannot cross the blood-brain barrier[155]. In contrast, the natural form of the vitamin, 5-methylTHF, is transported through the choroid plexus into the cerebrospinal fluid via RFC. 5-methylTHF is considered a natural neuroligand capable of binding to GRIK2/GluR6, whose locus has been associated with autism[153,156]. Injecting 5-methylTHF into the brain has been shown to reproduce seizures similar to those produced by kainic acid and cause long-term brain damage, but unlike kainic acid, it does not exhibit any local neurotoxicity. 5-methylTHF-dependent Hcy methylation also impacts the glutamatergic nervous system since Hcy can act as neurotoxicant by binding to a subgroup of excitatory glutamate receptors, the N-methyl-D-aspartate (NMDA) receptor, involved in synaptic transmission. Given the clear evidence that glutamate dysregulation is associated

with autism, the excitotoxicity of glutamate by folic acid and Hcy, and 5-methylTHF with the kainic acid receptor draws attention to the study of folate metabolism as a possible key factor in the onset of ASD, especially given the already demonstrated impact it has on congenital defects such as spina bifida[157]. In fact, during embryogenesis, folate deficiency and polymorphisms of the genes involved in its cycle can lead to neural tube defects[158]. DHFR is the only enzyme responsible for maintaining the reduced state of the vitamin B6 and, together with MTHFR, for de novo synthesis of methionine and thymine, essential for DNA expression and processing[116]. In Western diets, there is a trend towards increased use of folic acid during pregnancy, leading to increased levels of unmetabolized folate during embryogenesis[104]. As already mentioned, DHFR is the crucial enzyme in maintaining optimal folate levels and literature reports that the *DHFR* 19bp variant may represent a significant risk factor for autism. Preliminary data show that this polymorphism interacts with other important genes in the folate cycle, such as MTHFR, in particular with the C677T *MTHFR* SNP, but further studies are needed to confirm these data[150].

CBS: gene and function

The CBS gene is located on chromosome 21 (21q22.3) and comprises a total of 23 exons[159]. The resulting protein, cystathionine β-synthase, is made up of 551 amino acids and consists of 4 subunits, each of 63 kDa, forming a homotetramer[160]. Each subunit binds the two enzyme substrates, Hcy and serine, to catalyze the irreversible conversion of Hcy into cysteine, through the intermediate cystathionine. This conversion requires vitamin B6 as an essential cofactor and is the first reaction in the transsulfuration pathway closely linked to the methionine cycle, from which it extracts Hcy[161,162]. Elevated Hcy levels lead to redox imbalances and oxidative stress, resulting in the release of free radicals, whereas cysteine has antioxidant properties, protecting biological macromolecules like DNA, lipids, and proteins from oxidative damage. Increased Hcy, oxidative stress, including mitochondrial dysfunction, and altered thiol metabolism are among the primary metabolic issues associated with autism disorders[163–165]. These alterations have also been found in mothers of autistic children[166,167].

Oxidative stress or thiol metabolism is closely related to DNA methylation, imprinting, and genome integrity[168,169]. The junction point is represented by the recycling of Hcy, which involves three metabolic pathways and numerous cofactors, mainly B-group vitamins, and

Zn. The first pathway is the folate cycle, which requires vitamins B3, B9, and B12[168] and which is linked to the transsulfuration pathway, in which CBS requires vitamin B6 as a cofactor to convert Hcy into cysteine, one of the three components of glutathione. In fact, it is first conjugated with glutamate by the enzyme glutamate-cysteine ligase to form γ-glutamylcysteine, and subsequently, through the enzyme glutathione synthetase, a glycine is added, producing glutathione. This powerful antioxidant serves as a universal endogenous protector against DNA damage caused by oxidative stress. The third pathway of Hcy recycling occurs mainly in the liver, using BHMT and producing methionine, which reenters the remethylation cycle, where AdoMet, a universal methylation agent, is generated[108]. A wide range of disorders, including neuropsychiatric disorders, autism, and cognitive decline, are associated with elevated homocysteine levels in biological fluids[170].

A deficiency of CBS leads to classical homocystinuria, a hereditary human disease characterized by vascular disorders (thromboembolism), skeletal abnormalities (osteoporosis), psychiatric disturbances, seizures, and mental retardation. Furthermore, alterations in the metabolism of Hcy and methionine have been associated with neurodegenerative diseases such as Alzheimer's disease and ASD[171,172]. A decrease in CBS activity increases the demand for cysteine, the amino acid that limits the rate of glutathione synthesis[173,174]. Low concentrations of cysteine have been observed in autistic children, leading to a reduction in total glutathione in favor of oxidized glutathione, suggesting greater vulnerability to oxidative stress[95]. Human oocytes and early embryos do not express CBS and only minimally express BHMT, which results in a limited capacity to eliminate high levels of Hcy, exposing them to free radicals[175,176]. These free radicals can alter the methylation of cytosine in DNA and, consequently, influence both imprinted and non-imprinted gene methylation[177,178]. It is important to note that the onset of ASD occurs after the first year of life, likely during the maturation and development phases of the central nervous system, and early detection of Hcy metabolic disorders could potentially prevent autism spectrum disorders[42]. It has been observed that supplementation of essential factors in older autistic children can help restore the correct metabolic profile but cannot reverse damage that has already occurred since nervous system development has already taken place[179].

It is known that the human *CBS* gene has a large number of mutations, most of which are missense in nature[159]. Three different nonsense mutants have also been identified, as well as insertion, deletion, and junction variants, some of which are of polymorphic nature[180,181]. A

common *CBS* gene polymorphism is the insertion of 68 bp (844ins68; rs876657421), which duplicates the 3¢ splice site of intron 7 and the 5′ end of exon 8[182] and has been associated with an increased risk of schizophrenia[183]. The insertion frequency is estimated to be between 5 and 10% in Caucasians [180,184], absent among Asians [185], and much more prevalent among Blacks[186]. One insertion variant is believed to be linked to increased enzyme activity because it has been associated with reduced plasma homocysteine levels[187,188]. Studies on the association of the 844ins68 variant with schizophrenia have confirmed a lower insertion frequency in schizophrenic patients compared to controls[183]. In an attempt to associate other *CBS* gene polymorphisms with ASD, given its role in regulating Hcy, the results have revealed an association between the C699T variant (rs234706) and children with ASD. In particular, the genetic combination 699TT and 699CT was more frequent in children with ASD compared to controls[189]. Based on these findings, further investigations could identify more specific associations where there is already a proven implication of the *CBS* gene in methylation status, oxidative stress, and ASD.

Epigenetic regulatory networks associated with ASD: methylation DNA and *LINE-1*

Epigenetics encompasses all those mechanisms that can induce changes in gene expression patterns without altering the genomic DNA sequence. These changes can be hereditary and modulated by environmental factors, including lifestyle, exposure to chemicals, or diet. Along with histone post-translational modifications, DNA methylation is a common epigenetic mechanism in mammalian genomes that can act as an activator or repressor of gene expression[190]. DNA methylation involves DNA methyltransferase (DNMT) enzymes that mediate the transfer of a methyl group from S-adenosyl-methionine (AdoMet/SAM) to the cytosines in CpG islands commonly found in gene promoter regions[191].

Epigenetics can be used as a tool to study complex developmental diseases and to identify innovative therapeutic strategies. Several studies have highlighted DNA methylation profiles in ASD using tissues from various sources, including lymphoblastoid cell lines, peripheral blood, and post-mortem brain tissues[33,178,192,193]. Differentially methylated genomic regions that include genes associated with common molecular pathways of autism have been identified in brain tissues of individuals with ASD disorder[193,194]. Furthermore, a large-scale analysis using CpG island microarrays on lymphoblastoid cells has revealed different methylation profiles between monozygotic twins with an ASD diagnosis and their non-autistic siblings[33]. In particular, they noted different methylation status in two candidate genes for ASD, BCL-2 and RORA (Retinoic Acid Receptor-Related Orphan Receptor Alpha), correlating with reduced protein expression in the brain tissues of individuals with ASD[33]. This represents an example of how epigenetic changes in peripheral blood may reflect the methylation status and pathophysiological conditions of the brain. For this reason, peripheral tissues can be considered as a surrogate to identify candidate biomarkers for ASD.

It is known that the human genome is rich in non-coding regions that play an important role in gene regulation. The study of epigenetics, such as DNA methylation, has focused on the methylation status of these regions[195]. Non-coding regions subject to epigenetic modifications include the Long Interspersed Element-1 (*LINE-1*), which is an autonomous mobile element that constitutes 17% of the human genome and belongs to the group of non-long terminal repeat (non-LTR) retrotransposons[196]. This transposable element consists of a 5′-UTR with an internal promoter, two open reading frames (ORF1 and ORF2), and a poly(A) sequence at the 3′-UTR[197]. *LINE-1* is capable of retrotransposing to a new position in the genome, causing genomic instability, duplications, deletions, or insertions of its

sequence at the target site, thereby altering gene expression[198]. The *LINE-1* promoter region contains CpG residues whose cytosines undergo epigenetic modifications, and the degree of *LINE-1* methylation is correlated with retrotransposon expression. Methylated CpG residues in *LINE-1* are recognized by the Methyl-CpG Binding Protein 2 (MeCP2), which represses *LINE-1* transcription and retrotransposition. Mutations in MeCP2 have been associated with Rett syndrome, a developmental disorder with autistic phenotypes[199].

Several studies have associated *LINE-1* with neuronal differentiation; its promoter, in fact, is activated through the canonical Wnt-signaling pathway, also associated with neural stem cell transition, neurogenesis, and neural progenitor survival[200,201]. Lower levels of *LINE-1* methylation have been detected in the brain tissues of individuals with ASD carrying the 15q11-q13 duplication[202], a common copy number variant associated with ASD. Specifically, methylation of the 5′-UTR, ORF1, and ORF2 was reduced in ASD compared to healthy controls. Compelling evidence suggests that aberrant DNA methylation profiles of *LINE-1* and *Alu* elements are linked to various diseases, including ASD, pre-symptomatic dementia in type 2 diabetes, and chronic lymphocytic leukemia. Whole-genome sequencing of ASD individuals' brain revealed that *LINE-1* and *Alu* insertions are present in greater amounts than in normal developmental brain tissues[203]. Furthermore, a recent study associated methylation of *LINE-1* and *Alu* elements with gene expression in the blood transcriptome, implying that these transposable elements can influence gene expression in ASD[204]. By integrating methylation analysis with transcriptome analysis, it was in fact possible to identify *LINE-1* and *Alu* target genes differentially expressed in multiple ASD cohorts[204,205]. It is essential to emphasize that ASD's heterogeneity masks differences in DNA methylation that are instead highlighted in subanalyses of ASD phenotypes. Indeed, studies conducted on lymphoblastoid cells and brain tissues reveal no differences in *LINE-1* methylation between heterogeneous ASD and control groups, while differences emerge when clinical phenotypes of the disorder and genetic variant (such as 16p11.2del and CHD8) are taken into consideration[204]. Analyses revealed changes in *LINE-1* methylation in a locus- and family-specific manner, depending on the ASD cohort examined[65,206,207]. Furthermore, it has been observed that the degree of methylation in peripheral blood reflects both that present in brain tissue samples and environmental exposure[204]. For this reason, the identification of global DNA methylation signatures via *LINE-1* could have clinical potential and be used as non-invasive biomarkers.

DNA methyltransferase (DNMT): player in DNA methylation and role in ASD

DNA methylation is the epigenetic mechanism crucial for essential processes in living organisms. It is employed to silence DNA fragments or entire chromosomes, program cell differentiation, and prevent errors in cellular DNA segregation. The decision to methylate DNA is crucial for tissue-specific gene expression and the temporal sequence of developmental stages. The highly conserved family of enzymes playing a significant role in this epigenetic mechanism is DNA methyltransferases (DNMTs)[208]. DNMTs are essential in the evolution of living organisms as they catalyze DNA methylation, a mechanism conserved from archaea to eukaryotes, from fertilization through every developmental stage, and from the early stages of cancer to metastasis. The DNMTs family acts in concert with other epigenetic factors and serves as the primary regulators of gene transcription. Due to their critical function, various mechanisms regulate the expression of DNMTs, many of which, with inhibitory function such as non-coding RNAs regulating DNMT mRNA expression or the overexpression of inactive DNMT isoforms produced by alternative splicing, have been described in all stages of cancer, from onset to metastasis[209]. For this reason, compounds inhibiting methylation have been combined with chemotherapy for cancer treatment[209]. DNMTs catalyze the transfer of the methyl group from the cofactor AdoMet to the C5 position of cytosine[210]. This process results in the creation of 5-methylcytosine on DNA, releasing AdoHcy, which can accept a new methyl group. This methylation occurs in cytosine residues, primarily in CG dinucleotides distributed mainly in repetitive DNA, transposons, and retroviruses, as well as in CG islands. The latter are regions at least 550 bp long, found in the promoters of approximately 70% of human genes, including housekeeping genes and tissue-specific genes[210]. Methylation of the DNA in the gene promoter region leads to the repression of transcriptional activity. Active genes typically exhibit hypomethylation near the transcription start site, while they are hypermethylated within the gene body, increasing gene expression.

In mammals, three active DNA methyltransferases exist: DNMT1, DNMT3A, and DNMT3B, along with a catalytically inactive related protein called DNMT3L[210]. DNMT1 was the first enzyme identified with DNA methyltransferase activity and shows a preference for hemimethylated DNA over unmethylated DNA. It functions as a maintenance methyltransferase, methylating hemimethylated DNA, localizing to DNA replication foci during the S phase of the cell cycle to maintain epigenetic memory in cells. In addition to its

primary maintenance role, DNMT1 may support DNMT3A and DNMT3B involved in de novo methylation by utilizing hemimethylated CG sites generated by DNMT3 enzymes as substrates. The DNMT3 gene is a paralog that has duplicated several times, generating three isoforms: DNMT3A, DNMT3B, and DNMT3L. These proteins interact with each other, forming a complex where DNMT3L enhances the catalytic activity of the other two isoforms[211]. The main functions of DNMTs in mammals include the repression of repeated sequences, development, gene regulation, epigenetic memory, and cancer progression.

DNA methylation patterns in the early stages of mammalian development and in germ cells are established by DNMT3A and DNMT3B, considered de novo DNMTs because they do not show significant preference between methylated and unmethylated DNA[210]. In addition to this primary function, they also play a role in maintaining DNA methylation in heterochromatin regions. DNMT1 is expressed in proliferating cells and somatic tissues. Mutations in the DNMT1 gene in mice lead to global DNA hypomethylation. Conversely, DNMT3A and DNMT3B are enzymes under regulated in somatic cells and highly expressed in embryonic cells, early embryos, and germ cells[210]. In these cells, DNMT3L functions as a regulatory factor without an active catalytic site.

Preliminary studies have suggested an association between genetic variations in DNMTs, particularly DNMT1, and the degree of *LINE-1* sequence methylation. Additionally, data suggest a link between *LINE-1* methylation in peripheral blood and circulating AdoMet levels in healthy individuals[212]. DNMT1 polymorphism could modify this link in the presence of low plasma AdoMet levels. Unlike studies conducted on DNA methylation as an epigenetic mechanism related to ASD, there are few studies exploring the association of common variants in DNMT genes, which play a central role in methylation control, with ASD. One study conducted a screening of DNMT polymorphisms in autistic patients to identify a potential association between genetic variations in DNMTs and ASD[213]. Specifically, they demonstrated that DNMT1 G/A rs14018707 and C/A rs10423341, as well as DNMT3A G/A rs2289195, are significantly associated at both the genotypic and allelic levels with ASD[213]. Further studies are needed to delve into the potential role of DNMT genetic variants in ASD susceptibility, considering their contribution to DNA methylation as well.

Aims of the study

Autism spectrum disorder (ASD) is a highly heritable and heterogeneous neurodevelopmental disorder that presents consistent cognitive characteristics and commonly co-occurs with other conditions. It is due to a combination of genetic and environmental factors which often lead to a compromised and delayed diagnosis. Manifestations of autism include alterations in communication and social interaction, sensory abnormalities, repetitive behaviors, and varying levels of intellectual disability. In addition to these main symptoms, co-occurring psychiatric or neurological disorders are common in people with autism, including hyperactivity and attention disorders, anxiety, depression, and epilepsy[214].

Given the wide inter- and intra-individual heterogeneity of ASD, the etiology is probably multifactorial, characterized by a complex integration of genetic, epigenetic, and environmental factors that play a significant role by affecting the two sexes differently[21]. Various risk factors have been explored to understand the causes of this broad spectrum of disorders.

The numerous genes identified as risk factors for ASD encode proteins that function in chromatin remodelling, transcriptional regulation, or synaptic architecture and function[25,27]. However, not all children with a predisposing genotype develop ASD, suggesting that genetic factors alone cannot explain the onset of the pathology. For this reason, it is imperative to further explore the role of environmental factors that interact with the genome through epigenetic mechanisms that influence gene expression and neurotypical development.

Maternal immune dysregulation events during gestation represent high risk factors for autism[65,215]. In cohort studies it has been highlighted that infections during pregnancy can have a significant impact on neurodevelopmental processes, causing immune disorders and cytokine production in the mother. These factors contributing to neurodevelopmental disorders may be related to an altered immune state characterized by activation of microglia in various parts of the central nervous system, phenomena related to genetic and epigenetic effects, alterations in neurotransmitters, and abnormalities in signalling pathways and endocrine alterations. Studies in human and animal models have suggested that during crucial periods for neurological development and immune system development, inflammatory, genetic, and epigenetic mechanisms are critical[53].

Based on these premises, we conducted an extensive study on a group of patients diagnosed with ASD, analysing a combination of biochemical, genetic, and epigenetic factors. The aim was to identify the best combination of components predisposing to the disorder, with particular attention to the severity of ASD. Indeed, this disorder is characterized by a wide range of symptoms and clinical features that must be taken into consideration to characterize and identify ASD subgroups, thus enabling personalized therapies.

For the biochemical aspect, we aimed to investigate the role of the inflammatory system in ASD, particularly systemic cytokine release, by analysing the plasma of patients with ASD and comparing it with a group of age-matched and genetically related individuals to minimize sample heterogeneity. Special attention was paid to intra-group comparisons of patients, considering different phenotypes and symptom severity. For this reason, we stratified the ASD group based on the main clinical characteristics and comorbidities observed in individuals with ASD.

To identify the genetic component predisposing to ASD, we analysed polymorphisms of the main genes involved in the folate cycle, such as *MTHFR* C677T (rs1801133), *MTHFR* A1298C (rs1801131), *DHFR* 19bp (rs70991108), and *CBS* 68bp (rs876657421), since the folate cycle plays a recognized essential role in neuronal development and ASD and is directly linked to the remethylation cycle indispensable for epigenetic mechanisms. Alterations in the latter can lead to genomic instability and alterations in gene expression, contributing to neurodevelopmental disorders such as ASD. For this reason, we also analysed polymorphisms of *DNMTs* genes such as *DNMT1* rs2228612, *DNMT3A* rs734693, *DNMT3B* rs1569686, and *DNMT3L* rs2070565.

Finally, we explored global DNA methylation through the analysis of *LINE-1* promoter methylation, conducting intra-case analyses as well as analyses among family members recruited in the study. Considering the multifactorial etiology of ASD, we used a multicomponent approach by conducting multivariate analyses to highlight combinations of variables that may contribute to autism development, thus enabling the identification of preventive strategies and targeted therapeutic interventions.

Materials and Methods

Study population

For this retrospective study, various family units were recruited, comprising 75 patients with confirmed ASD diagnoses and 160 subjects with typical development, to explore the association of cytokine levels, genetic factors, and epigenetics with ASD. The study population included family units composed of the ASD-affected patient, both biological parents, and any siblings.

The study was conducted in the laboratory of Professor Donato Gemmati and Professor Veronica Tisato at the Department of Translational Medicine of Romagna at the University of Ferrara. The study followed the guidelines established by the Declaration of Helsinki, and all procedures were approved by the Ethics Committee of the Pederzoli-Peschiera Hospital in Verona. Participants in the study provided informed consent.

Sample collection and processing

The project involved collecting blood samples from peripheral venous blood into 3 mL sodium citrate tubes. Blood samples were processed to separate plasma from the cellular component of the blood. The sodium citrate tubes were centrifuged at 2000 x g for 10 minutes at room temperature (RT) to collect platelet-poor plasma (PPP). The PPP was aliquoted into 500 µL portions and stored at -80°C for subsequent cytokine analysis.

In contrast, the cellular component of the blood was stored at -20°C, awaiting DNA extraction and subsequent genetic and epigenetic analysis for the present study.

Cytokine levels measurement

For the cytokine analysis, a multiplex immunological assay was employed, utilizing magnetic microspheres to simultaneously analyze multiple cytokine and chemokine biomarkers. Each magnetic sphere presents an analyte-specific antibody that allows for the recognition of different cytokines in the sample. Additionally, each sphere is fluorescently encoded through varying ratios of two fluorophores, resulting in a unique fluorescent signature for each sphere.

The analytes are recognized using a sandwich immunoassay, wherein the secondary antibody is covalently conjugated with a fluorophore enabling the quantification of the bound analyte. This technology utilizes a flow cytometry-based system where each sphere flows through the cytometer capillary. The spheres are identified using a red laser (635 nm) that recognizes the fluorescent signature of the sphere, and a green laser (525 nm) that quantifies the bound analyte sandwiched to the bead (figure 4).

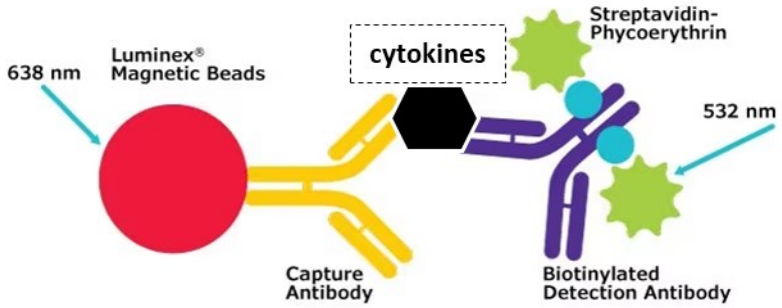


Figure 4. The immunological recognition complex between analyte and antibody is based on Luminex technology. The analyte can be the cytokine/chemokine recognized by the capture antibody. The primary antibody is bound to Luminex magnetic beads and incubated with the analyte for 16 hours. To create the sandwich, the analyte is bound by the biotinylated detection antibody. Finally, the complex is completed when streptavidin-phycoerythrin is added, which binds to the biotin of the secondary antibody. The excitation wavelengths of the fluorophores present in the immunological recognition complex of Luminex technology (Merck) are 638 nm and 532 nm. Figure adapted from Merck Company with modifications highlighting the analyte (cytokines).

For the study, a panel of cytokines from Millipore was selected, allowing the measurement of the concentration of 19 cytokines: G-CSF, GM-CSF, IFN-γ, IL-1β, IL-1RA, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-17A, MCP-1, and TNF-α, in plasma samples. Each kit included a reference standard for creating the calibration curve (7 points of the calibration curve through 1:5 dilution) to enable quantitative analysis

and two quality controls (QCs), i.e., high and low dilutions of recombinant proteins for each analyte for assay validation. Additionally, an internal sample was added to each test as a control to assess assay reproducibility.

The first step of the technique involved incubating 25 µL of PPP with the mix of primary antibodies covalently bound to the magnetic beads for 16 hours with constant agitation. Specifically, the sample wells (96-well plate) consisted of 25 µL of PPP, 25 µL of bead-antibody mix, and 25 µL of assay buffer. In contrast, the calibration curve and quality control wells contained 25 µL of standard and QCs (high and low), respectively. Using fluorescent molecules, the entire procedure was performed in the dark. After primary incubation, 5 washes of 200 µL each were performed with wash buffer using a magnetic plate to preserve the beads and the bound sample during washing. To enable sandwich immunological recognition, a mix of biotinylated secondary antibodies (25 µL per well) specific for the 11 analytes examined in the PPP was added. The secondary incubation lasted for 1 hour at room temperature (20-25°C), with constant agitation. Subsequently, 25 µL of Streptavidin-Phycoerythrin was added to each well containing 25 µL of the secondary antibody cocktail. A 30-minute incubation at room temperature allowed for high-affinity recognition typical of streptavidin with the biotin conjugated to the secondary antibody present in the well. The wells of the incubation plate were washed 5 times with 200 µL each of wash buffer to eliminate nonspecific components. Finally, 150 µL of drive fluid was added to each well to resuspend the immunological sandwich and allow reading of the plate on the MAGPIX® instrument with xPONENT® software. Upon completion of the reading, the analysis allowed determination of the concentration expressed in pg/mL for only 11 cytokines out of the 19 analyzed due to under-detection. The cytokines detectable in our samples were: G-CSF, IL-1β, IL-1RA, IL-4, IL-5, IL-7, IL-8, IL-10, IL-12(p40), MCP-1, and TNF-α.

DNA Extraction and Purification

Genomic DNA was extracted from blood samples using the EZ1&2 DNA Blood Kit and the BioRobot EZ1 instrument from Qiagen. A volume of 350 µL of blood was used for genomic DNA extraction. In a single step, nucleated blood cells are lysed, and DNA is isolated by binding to the silica surface of the particles in a chaotropic saline solution. In the subsequent phase, the particles are separated from the lysates using a magnet, which also enables two successive washes without sample loss. Finally, the DNA was eluted in 100 µL of nuclease-free water for subsequent quantification (Figure 5).

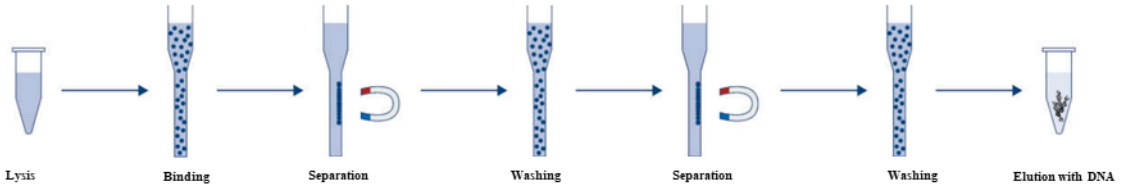


Figure 5. Automated DNA Extraction Process. The first phase of automated extraction involves lysing the cellular and nuclear membranes, releasing nucleic acids. Subsequently, DNA is captured by silica-based magnetic beads. Due to their magnetic nature, DNA can be isolated in subsequent washing steps and finally eluted in the elution buffer. Figure adapted from Qiagen Company with modifications highlighting the extraction steps and DNA in the final eluate.

Quantification and verification of the purified DNA were performed using the UV spectrophotometer, Agilent Cary 60. Absorption at 260 nm is characteristic of nucleotides, while absorption at 280 nm measures protein residues present in the extracted sample. To ensure the purity of the DNA sample and facilitate quantification, the ratio of A260/A280 was measured. DNA samples with an A260/A280 ratio between 1.8 and 2.0 were validated for subsequent genetic amplification and detection.

Genetic Investigations

For the study of genetic factors implicated in ASD, 8 polymorphisms in 7 genes were selected, whose products are the main actors in one-carbon metabolism. Specifically, 4 polymorphisms of 3 genes involved in the folate cycle were selected: *MTHFR* C677T (rs1801133), *MTHFR* A1298C (rs1801131), *DHFR* 19bp (rs70991108), and *CBS* 68bp (rs876657421). Additionally, 4 polymorphisms of DNMTs implicated in the methylation

mechanism were studied: *DNMT1* rs2228612, *DNMT3A* rs734693, *DNMT3B* rs1569686, and *DNMT3L* rs2070565.

Various DNA amplification techniques were used. As shown in Table 1, the *DHFR* 19bp (rs70991108) and *CBS* 68bp (rs876657421) polymorphisms were detected via qualitative PCR and electrophoretic separation on agarose gel. In contrast, the polymorphisms *DNMT1* rs2228612, *DNMT3A* rs734693, *DNMT3B* rs1569686, and *DNMT3L* rs2070565 were analyzed using Real-Time PCR and genotyping assays. Finally, the *MTHFR* C677T (rs1801133) and A1298C (rs1801131) gene polymorphisms were identified through pyrosequencing.

Table 1. Information of polymorphisms and detection methods

Gene Symbol	Gene Name	Chr	Ref. Sequence	Nt change	AA change	Method
MTHFR	Methylenetetrahydrofolate reductase	1	rs1801133	C677T	A223V	Pyrosequencing
MTHFR	Methylenetetrahydrofolate reductase	1	rs1801131	A1298C	E429A	Pyrosequencing
DHFR	Dihydrofolate reductase	5	rs70991108	19 bp ins/del	NA	Electrophoretic separation
CBS	Cystathionine β-synthase	21	rs876657421	68 bp ins/del	NA	Electrophoretic separation
DNMT1	DNA methyltransferase 1	19	rs2228612	T979C	I206V	Real-Time PCR
DNMT3A	DNA methyltransferase 3 alpha	2	rs734693	C>T	Intron Enhancer	Real-Time PCR
DNMT3B	DNA methyltransferase 3 beta	20	rs1569686	G>T	Promoter/ Regulatory region	Real-Time PCR
DNMT3L	DNA methyltransferase 3 like	21	rs2070565	T>C	Splicing site	Real-Time PCR

NA: not available.

PCR and Electrophoretic separation

To genotype the polymorphisms of the *MTHFR* C677T (rs1801133), *MTHFR* A1298C (rs1801131), *DHFR* 19bp (rs70991108), and *CBS* 68bp (rs876657421) genes, a qualitative polymerase chain reaction (PCR) was performed. The thermocycler used in this study was the SureCycler 8800 instrument from Agilent Technologies.

Specific primer pairs were designed for each polymorphism, and optimal temperature cycles were set to maximize the reaction yield. The primer sequences, different reaction mixes, and temperature cycles used are detailed in Table 2. For the genotyping of the folate cycle

polymorphisms, 150 ng of purified DNA sample were used in a 40 µL PCR reaction volume, and both a positive and a negative control were included in each PCR setup. A DNA sample with a known genotype served as the positive control, while a PCR solution containing all essential elements except template DNA served as the negative control to detect DNA contamination.

Table 2. Information and PCR conditions for genes in the folate cycle

Gene	Ref. Sequence	Primers	Mix	Cycle	DNA Fragment
MTHFR C677T	rs1801133	FW: 5′-ATCCCTATTGGCAGGTTACCC-3′ RV: 5′-Biosg AAAGAAAAGCTGCGTGATGATGA-3′	Buffer 1X Taq:0.04U/µL dNTPs: 0.2mM FW:0.8µM RV: 0.8µM	95°C 4min (95°C 30sec 54°C 30sec 72°C 1min) 35x 72°C 10min	121 bp
MTHFR A1298C	rs1801131	FW: 5′-CTGCCCTCTGTCAGGAGTGT-3′ RV: 5′-Biosg-CATTCCGGTTTGGTTCTCC-3′	Buffer: 1X Taq:0.05 U/µL dNTPs: 0.2mM FW:0.6 µM RV:0.9 µM	94°C 4min (95°C 30sec 56.5°C 15sec 72°C 45sec) 35x 72°C 4min	222 bp
DHFR	rs70991108	FW_1: 5′- CCCAGGTACCCCGACCGTGG-3′ FW_2: 5′- GAGTCGGCCACCCCGACCGT-3′ RV: 5′-AAAAGGGGAATCCAGTCGG-3′	Buffer: 1X Taq:0.03U/µL dNTPs: 0.2mM FW_1: 0.67µM FW_2: 0.67µM RV: 0.67µM	94°C 3min (94°C 55sec 60°C 35sec 72°C 35sec) 33x 72°C 10min	92 bp (del) 113 bp (ins)
CBS	rs876657421	FW: 5′-GTTGTTAACGGCGGTATTGG -3′ RV: 5′- GTTGTCTGCTCCGTCTGGTT-3′	Buffer: 1X Taq: 0.05U/µL dNTPs: 0.2mM FW: 1µM RV: 1µM	95°C 3min (95°C 45sec 58°C 40sec 72°C 40sec) 35x 72°C 5min	171 bp (del) 239 bp (ins)

Biosg: Biotin modification for pyrosequencing; Taq: Thermus Aquaticus Polymerase (Taq DNA polymerase, Roche); dNTPs: deoxyribonucleotide TriphosPhates (Promega), FW and RV: forward and reverse primers (IDT, Integrated DNA Technologies).

To verify the correct amplification of the DNA sequence of interest, agarose gel electrophoresis was performed. This technique was utilized to assess the purification of the amplified sequence via PCR and, in specific cases, to determine genotypes, such as in the polymorphisms of the *DHFR* 19bp (rs70991108) and *CBS* 68bp (rs876657421) genes. Genotype determination was feasible because these polymorphisms involve insertions and

deletions of specific sequences (see Table 1), resulting in bands of distinct lengths after PCR (see Table 2).

For the preparation of the agarose gel, 4% agarose and Tris Borate EDTA (TBE) buffer at pH 8 were used, consisting of 30 mg/mL Tris Base, 5.52 mg/mL Boric Acid, and 0.01M EDTA pH 8. Electrophoresis was conducted in TBE buffer at 100 V for 45 minutes or until complete resolution of the bands. Amplified DNA fragments were visualized using the UV lamp of the Axygen® gel documentation system (Corning) with GelRed Nucleic Acid Dye (Biotium), a DNA dye included during agarose gel preparation.

A 5 µL volume of the PCR reaction (total volume: 40 µL) was loaded into the agarose well to verify the correct amplification of the sequence of interest, facilitate pyrosequencing (refer to "Bisulfite Pyrosequencing of blood-derived DNA sample" paragraph), and determine genotypes, such as those for the *DHFR* gene illustrated in Figure 6.

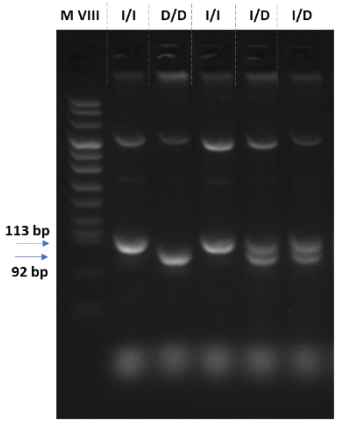


Figure 6. Electrophoretic run on a 4% agarose gel of the *DHFR* gene amplicon (rs70991108). DNA fragments from five different subjects separated by electrophoresis are depicted. Genotype assignment for the *DHFR* gene was conducted by identifying the insertion (113bp) and deletion (92bp) fragments. Arrows indicate the bands used for genotypic assignment. M VIII: DNA molecular weight Marker VIII (Sigma-Aldrich); I: sequence insertion; D: sequence deletion.

Pyrosequencing for SNP Genotyping

Pyrosequencing is a DNA sequencing method that utilizes bioluminescence generated during DNA synthesis. Each incorporated nucleotide emits a light signal, which is recorded and converted into a nucleotide sequence. This method is employed in next-generation sequencing for SNP genotyping, as well as for identifying bacteria, viruses, or determining the degree of methylation in nucleotide sequences.

The DNA strand used as a template for pyrosequencing is initially amplified via PCR (see Table 2 for information on PCR reactions). This step is crucial for incorporating a biotinylated primer, enabling the selection of the DNA strand for sequencing. Subsequently, the biotinylated DNA strand is immobilized on streptavidin-coated beads through the avidin-biotin linkage.

Sequencing in pyrosequencing relies on DNA polymerase I, which adds nucleotides to the sequencing primer that are complementary to the template strand, while the dNTPs (dATPαS, dCTP, dGTP, and dTTP) are dispensed sequentially one at a time. Each incorporation of a nucleotide generates pyrophosphate (PPi) (Equation 1, Figure 7). ATP sulfurylase enzyme utilizes PPi to produce equimolar ATP (Equation 2, Figure 7). Luciferase then uses the generated ATP to oxidize luciferin to oxyluciferin and emit light (Equation 3, Figure 7).

To complete the cycle, any unincorporated dNTPs and residual ATP are degraded by apyrase, according to the equations: dNTP → dNDP + dNMP + Pi; ATP → ADP + AMP + Pi (Equation 4, Figure 7). Once degradation is complete, another nucleotide can be dispensed into the reaction. Notably, dATP is replaced with dATPαS to ensure recognition only by DNA polymerase I and not by luciferase.

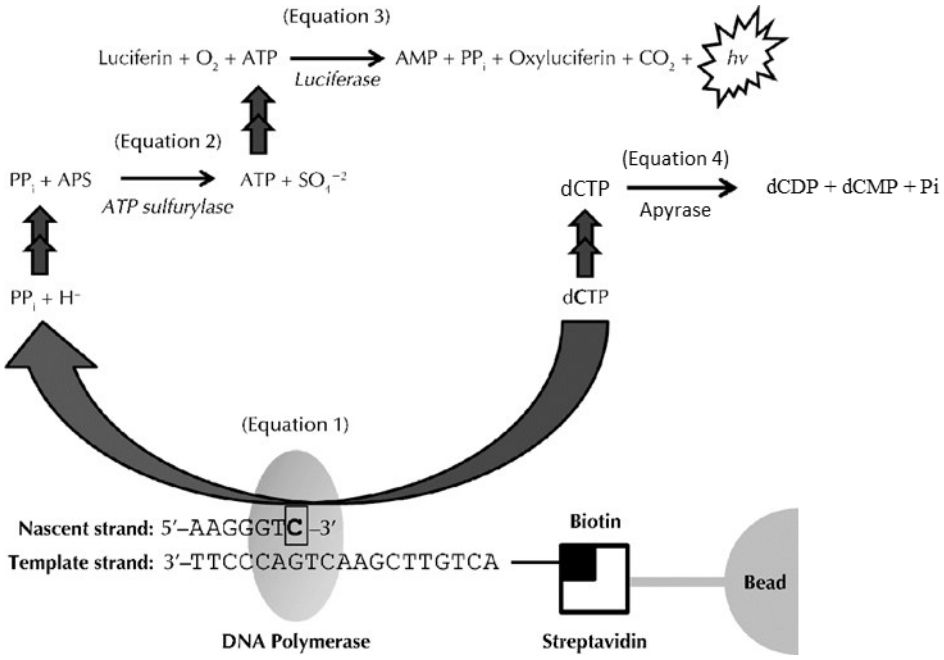


Figure 7: Chemical reactions of pyrosequencing. When the correct deoxynucleotide triphosphate (dNTP) is incorporated into the DNA strand being synthesized, the resulting inorganic pyrophosphate (PPi) from the condensation reaction initiates a cascade of enzyme reactions, culminating in the emission of light (hv). A hydrogen ion (H+) is also released alongside PPi. The equation numbers correspond to those listed in the introduction text. Abbreviations: AMP, adenosine monophosphate; APS, adenosine 5′-phosphosulfate; ATP, adenosine triphosphate; CO_2, carbon dioxide; O_2, oxygen; SO_4^{-2}, sulfate. Figure adapted from Harrington *et al.* 2013 with modifications showing also the apyrase reaction[216].

SNP genotyping was performed using the PyroMark ID Q96 instrument (Biotage), with 40 µL of PCR product used for each reaction. The pyrosequencing methodology can be divided into four phases:

I. Immobilization Phase: The PCR product is immobilized onto streptavidin-coated microspheres. An immobilization solution was prepared by mixing 37 µL of Binding Buffer (Qiagen) with 3 µL of Streptavidin Sepharose high performance (Cytiva) for each sample analysed. In a 96-well plate, the immobilization solution (40 µL) was added to each PCR product (40 µL) and incubated for 10 minutes at RT with vigorous agitation. This incubation allows the biotin in the incorporated primer to bind to streptavidin on the microsphere, forming a microsphere/DNA-template complex.

II. Fragment Separation Phase: During this phase, the double-stranded DNA filament is denatured, and the biotinylated DNA strand is selected. Using the Vacuum Prep

Tool/Worktable system, the microsphere/DNA-template complex undergoes denaturation and washing. The Vacuum Prep Tool transfers this complex to a series of solutions positioned in separate wells on the Vacuum Prep Worktable. It is started by washing it with nuclease-free water to prepare it to absorb the immobilization solution from the plate on the Vacuum Prep Worktable. Once the microsphere/DNA complex is absorbed, the Vacuum Prep Tool is sequentially immersed in a solution containing 70% ethanol, followed by Denaturation Solution (Qiagen), and finally Wash Buffer (Qiagen). Each solution is incubated for 5 minutes. After absorbing all the liquid from each solution, the microsphere/DNA template complex is transferred to the Annealing solution for the next phase.

III. Primer Annealing Phase: In this phase, the sequencing primer binds to the DNA template, initiating the sequencing reaction. For each sample, an Annealing solution (40 µL) was prepared containing Annealing Buffer (Qiagen) and 2.25 µM of sequencing primer (purified by HPLC, IDT). The Annealing solution was incubated with the DNA template for 2 minutes at 80°C. SNP-specific sequencing primers were designed using PyroMark Assay Design 2.0. For instance, the sequencing primers for *MTHFR* C677T (rs1801133) and *MTHFR* A1298C (rs1801131) were 5′-GAAGGTGTCTGCGGG-3′ and 5′-GGAGCTGACCAGTGAA-3′, respectively.

IV. Sequencing Reaction Phase: Following the annealing phase, the samples are sequenced using the PyroMark ID Q96 instrument and PyroMark Software. In this phase, PyroMark Gold Q96 Reagent (Qiagen) is added to the sequencing reaction. It includes the substrate, enzyme mixture, and the necessary dNTPs, with volumes decided by the software based on the number of samples analyzed.

During DNA synthesis, incorporation of each nucleotide generates a light signal, which is captured as peaks in the resulting pyrogram. Figure 8 illustrates the pyrogram of the MTHFR C677T gene polymorphism (rs1801133), where light signal intensity (y-axis) is plotted against time (x-axis).

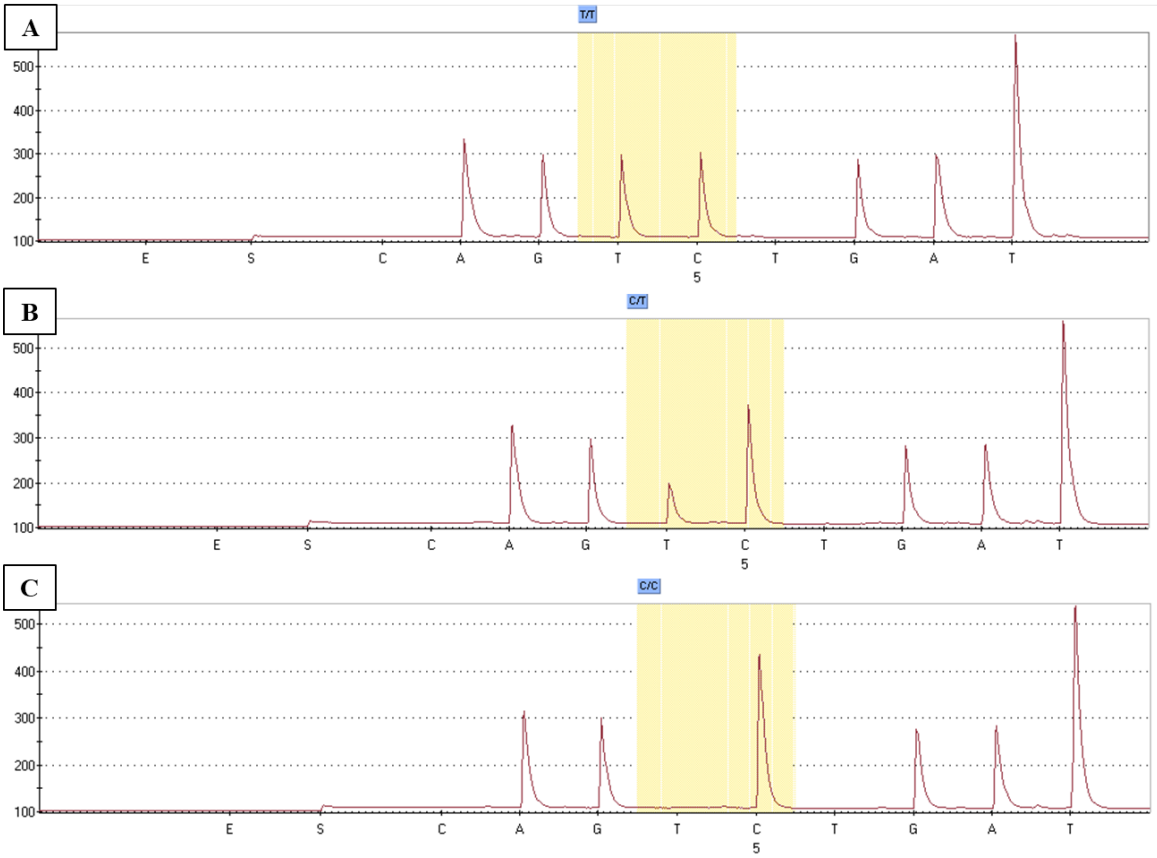


Figure 8. Pyrogram of *MTHFR* C677T gene (rs1301133). Pyrograms of three different samples are shown, illustrating the various genotyping conditions for the MTHFR gene polymorphism. The intensity of the light signal generated by the addition of dNTPs to the complementary sequence is indicated on the y-axis. The temporal sequence of dNTPs dispensed during pyrosequencing is plotted on the x-axis. The light signal threshold is identified after the substrate (S) is dispensed in the sequencing reaction, which occurs subsequent to the enzyme (E). **(A)** TT-genotype; **(B)** CT-genotype; **(C)** CC-genotype. The yellow area indicates polymorphic site.

Real-Time PCR for SNP Genotyping

For the genotyping of DNMT SNPs, including *DNMT1* rs2228612, *DNMT3A* rs734693, *DNMT3B* rs1569686, and *DNMT3L* rs2070565, Real-Time PCR was performed using the TaqMan® SNP Genotyping Assay by Applied Biosystems (ThermoFisher). This assay employs TaqMan® 5′-nuclease chemistry to amplify and detect specific SNPs in genomic DNA samples. The TaqMan® SNP Genotyping Assay includes forward and reverse primers and two probes that consist of target-specific oligonucleotides with a fluorescent dye attached to the 5′ end and a quencher attached to the 3′ end. The genotyping test determines the presence or absence of the polymorphism based on the fluorescence emitted by the allele-specific probe. Figure 9A illustrates two probes labelled with different fluorophores: VIC is attached to the allele-specific probe containing guanine (G), while FAM is attached to the second probe containing adenine (A). The signal from the fluorescent dye at the 5′ end of a TaqMan probe is quenched by a nonfluorescent quenchers (NFQ) at the 3′ end through fluorescence resonance energy transfer (FRET). In addition, any probes have TaqMan® minor groove binder (MGB), this modification at the 3′ end allows binding to the minor groove of the DNA helix, enhancing hybridization in the MGB probe-template complex.

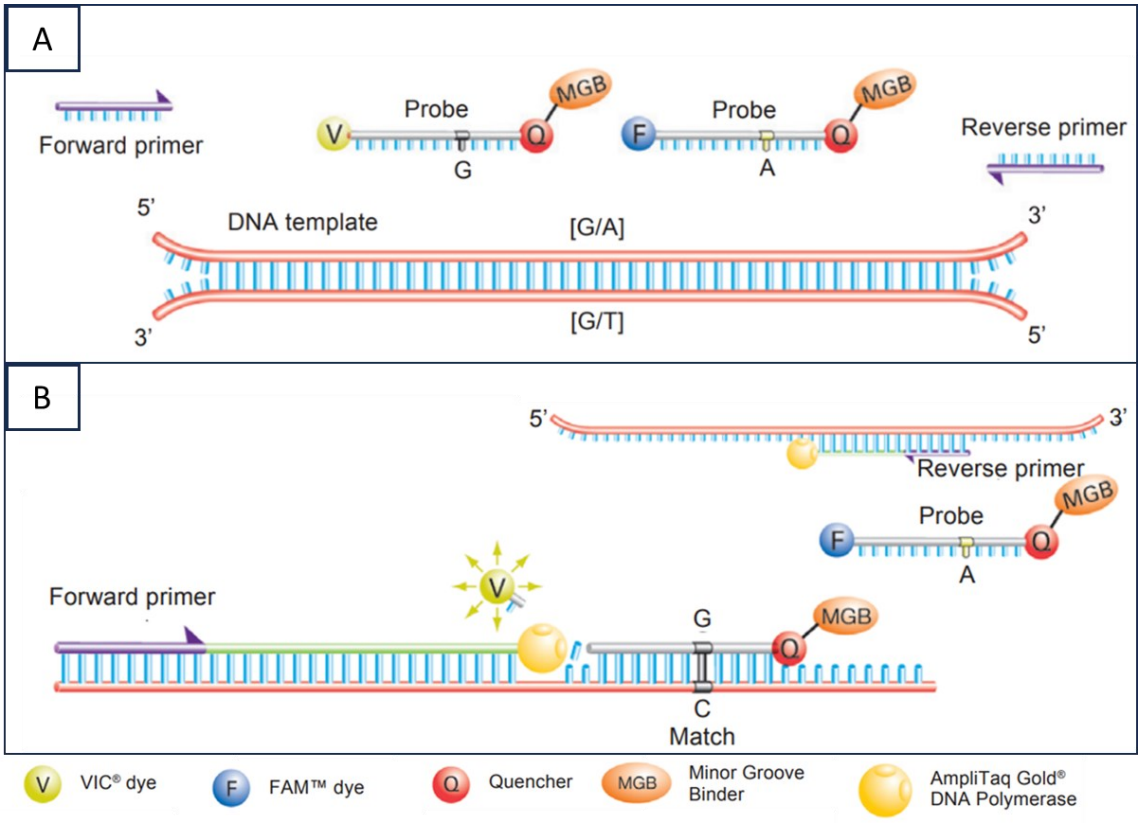


Figure 9. Real-Time qPCR technique. (**A**) Components of the TaqMan SNP Genotyping Assay and DNA template. (**B**) The probe has annealed to the template DNA, and polymerization results in the generation of a fluorescent signal. Figure adapted from ThermoFisher Company with modifications.

During PCR, the double-stranded DNA sequence undergoes denaturation, allowing the forward and reverse primers to hybridize to the separated strands. In this phase, the labelled MGB probes also hybridize to the allele-specific strands. During the extension phase of PCR, Taq polymerase uses the template strand to extend the unlabelled primer (Figure 9B). As Taq polymerase encounters the hybridized probe, it cleaves the 5′ end of the probe, separating the fluorophore from the quencher (Q). The qPCR instrument detects the fluorescence emitted by the fluorophore and determines the corresponding genotype. In samples from homozygous individuals, fluorescence from one of the two fluorescent dyes (FAM or VIC) is emitted. Conversely, samples from heterozygous individuals emit both fluorescent signals. With each PCR cycle, the emitted fluorescence intensifies as more dye molecules are released proportionally to the amount of synthesized amplicon.

For the genotyping of the 4 DNMT SNPs, 20 ng of purified DNA sample were used in a 10 µL PCR reaction volume. The reaction mix consisted of 5 µL of 2x TaqMan® Master Mix (ThermoFisher) and 0.5 µL of 20x TaqMan™ SNP Genotyping Assay (ThermoFisher). Each Real-Time PCR reaction included a negative control to detect any DNA contamination, and three positive controls were included, representing samples with known genotypes corresponding to the three possible genotype combinations for each SNP analyzed.

The Real-Time PCR conditions were as follows: initial denaturation at 95°C for 10 minutes (polymerase activation phase), followed by 40 cycles of denaturation at 95°C for 15 seconds (DNA denaturation phase) and annealing/extension at 60°C for 1 minute. The fluorescent signal was detected during the extension phase at 60°C and in the pre- and post-read phases using the QuantStudio™ 3 Real-Time PCR System (ThermoFisher). Genotyping analyses were performed using ThermoFisher Connect software.

Bisolfite Pyrosequencing of blood-derived DNA sample

DNA methylation levels were analyzed using pyrosequencing technology coupled with bisulfite conversion. This method enables the quantification of methylation at individual bases within a genomic locus and is widely regarded as the 'gold standard' for quantitative DNA methylation analysis. This epigenetic modification involves the addition of a methyl group to cytosine residues at CpG sites in genomic DNA. To detect these modifications, CpG sites are initially chemically treated with sodium bisulfite, converting unmethylated cytosine (C) to uracil (U), while methylated cytosine (5mC) remains unaltered due to protection by the methyl group. During subsequent PCR amplification, uracil is converted to thymine (T), whereas 5mC is amplified as cytosine (C), as illustrated in Figure 10.

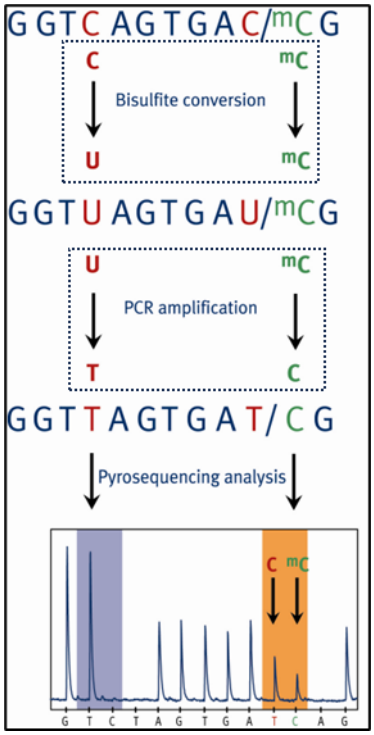


Figure 10. Principle of Bisulfite Pyrosequencing analysis. Unmethylated cytosine (C, marked in red) and methylated cytosine (5mC, marked in green) are distinguished through bisulfite treatment and PCR amplification. The ratio of C to 5mC at each CpG site is measured within the sequence context (peaks in orange columns). C bases not followed by G serve as controls for the bisulfite conversion step (blue column). Figure adapted from England et Pettersson, 2005, with modifications highlighting alterations in sequence bases [217].

This method distinguishes between 5mC and C during pyrosequencing. In the pyrogram, 5mC and C are represented by peaks C and T, respectively (see Figure 10). The peak height correlates with the number of methylated alleles at each CpG site. The methylation level at

each CpG site is calculated as the percentage of methylated alleles divided by the total number of methylated and unmethylated alleles.

To assess global DNA methylation in the studied population, bisulfite pyrosequencing of the *LINE-1* sequence was performed. Specifically, the methylation status of 5 CpG sites in the promoter region of the transposon (+306, +318, +321, +328, and +364) was analyzed.

500 ng of genomic DNA was converted using sodium bisulfite with the EpiTect 96 Bisulfite Kit (Qiagen). The conversion reaction for each sample consisted of 85 µL of Bisulfite Mix (Qiagen) + 35 µL of DNA Protect Buffer (Qiagen), making a total volume of 140 µL. Thermal conditions for the conversion included six phases: alternating between DNA denaturation for 5 minutes at 95°C, and incubation phases at 60°C for 25, 85, and 175 minutes, to facilitate the conversion of unmethylated cytosine to uracil.

Purification of the converted DNA sample was performed using a column-based method with buffers provided by the EpiTect 96 Bisulfite Kit (Qiagen), following the manufacturer's instructions. The purified DNA was eluted in 70 µL of Buffer EB (Qiagen) and stored at -20°C.

For *LINE-1* PCR amplification, the PyroMark PCR kit (Qiagen) was used with 9.28 µL of converted DNA in a total reaction volume of 40 µL. The PCR mix included 1x Master Mix, 1x Coral Load Concentrate, 0.5x Q-solution, and 0.4 µM of each primer (IDT). The forward primer sequence was 5′-TTTTGAGTTAGGTGTGGGATATA-3′ and the reverse primer sequence, containing a biotin residue crucial for pyrosequencing, was 5′-biotin-AAAATCAAAAAATTCCCTTTC-3′. A negative control was included in each PCR reaction to monitor for DNA contamination. Thermal cycling was performed using a SureCycler 8800 instrument (Agilent Technologies) with an initial activation step at 95°C for 15 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 15 seconds, extension at 72°C for 30 seconds, and a final extension phase at 72°C for 10 minutes.

To confirm the amplification of the *LINE-1* sequence, 5 µL of PCR product was loaded onto a 1.5% agarose gel in TBE buffer. Electrophoresis was conducted at 100 V for 30 minutes, and the 155 bp fragments were visualized using the Axygen Gel Documentation System (Corning). Figure 11 displays the resulting gel image.

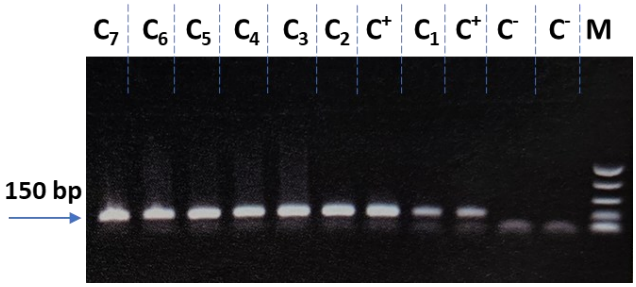


Figure 11. Electrophoretic separation on 1.5% agarose gel of the *LINE-1* sequence amplicons (150 bp). C_1 to C_7 represent different samples; C^+: positive control; C^-: negative control; M: DNA molecular weight PCR Marker (New England Biolabs). The arrow indicates the amplified sequence.

The residual PCR product (35 µL) was analyzed by pyrosequencing using CpG islands software 1.0 (Qiagen). Refer to the "Pyrosequencing for SNP Genotyping" section for details on the pyrosequencing procedure. The sequencing primer used was 5′-AGTTAGGTGTGGGATATAGT-3′, and the analysis sequence was TTYGTGGTGYGTYGTTTTTTAAGTYGGTTTGAAAAGYGTA. The dispensation order of dNTPs during the reaction was GTCGTGTAGTCTGTCGCTTATGTCGTGAATGTCGT (see Figure 12).

The methylation level at each sequenced CpG site was calculated as the percentage of methylated alleles divided by the total number of methylated and unmethylated alleles [C% = C/(C+T)]. The average global DNA methylation level analyzed in this study was determined using the methylation levels of the first 4 CpG sites within the *LINE-1* promoter region (+306, +318, +321, +328).

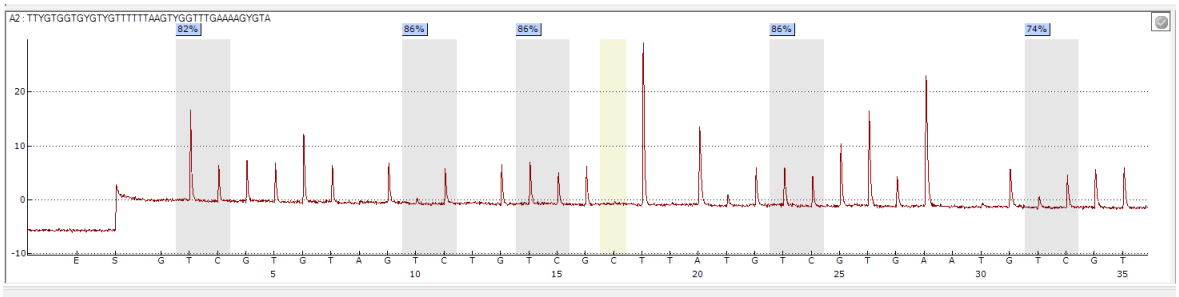


Figure 12. Pyrogram of the *LINE-1* sequence. The methylation percentage is depicted at the top (blue rectangles) for each CpG site analyzed. Cytosines (C) and thymines (T) are highlighted by gray zones. The intensity of the light signal recorded for each dNTP added during sequencing is indicated on the y-axis, while the dispensation of enzymes (E), substrates (S), and dNTPs over time is shown on the x-axis. The yellow area on the pyrogram represents the internal control for bisulfite conversion. The analysis sequence is displayed in the top left corner.

Statistical Analysis

PSS Statistics version 22 (SPSS Inc., Chicago, IL, USA) and MedCalc version 20.11.2 (MedCalc Software Ltd.) were used for the statistical analysis. All graphs presented in the results were generated using GraphPad Prism 8 (GraphPad Software, Inc., San Diego, California, USA). Variables were expressed as mean, standard deviation, and percentiles. Data were compared using the non-parametric Mann-Whitney U test, with p-values set at two-tailed for cytokine analyses and one-tailed for methylation levels, with values less than 0.05 considered statistically significant.

Pearson's correlation test was used for correlation analyses, and regression analysis was employed to compare correlations between different groups. Results were considered statistically significant if two-tailed p-values were < 0.05.

Principal component analysis (PCA) was used to analyze scalar and categorical variables. Scalar variables such as cytokine levels, methylation levels, and age were normalized for PCA using the formula: $(x - \mu) / SD$ [$Z = (x - \mu) / SD$]. Categorical variables including genotypes and clinical data were assigned scores of 1, 2, and 3 for common homozygotes, heterozygotes, and rare homozygotes, respectively. Clinical data were scored similarly to denote severity from mild to severe.

Sample adequacy was assessed using the Kaiser-Meyer-Olkin index (> 0.5) and Bartlett's test of sphericity (< 0.05). Principal components (PCs) explaining a total variance greater than 60% and with eigenvalues > 1 were selected. Variables with factor loadings greater than +0.3 or less than -0.3 were considered dominant for each PC. These multivariate analyses were applied to compare individuals with ASD and unaffected siblings, as well as within the ASD group using patient and available clinical data. Results were considered statistically significant if two-tailed p-values were < 0.05.

Results

The results chapter is divided into three subsections that analyse potential biochemical, epigenetic, and genetic factors implicated in ASD. As biochemical factors, the levels of cytokines in plasma samples have been examined. Conversely, for the epigenetic component, global DNA methylation has been explored, specifically the methylation of CpG islands in the *LINE-1* promoter sequence. For the genetic implications of the study, the main genes and polymorphisms related to methylation, such as the *DNMT*s genes (*DNMT1* rs2228612, *DNMT3A* rs734693, *DNMT3B* rs1569686, and *DNMT3L* rs2070565), and key genes involved in one-carbon metabolism, such as *MTHFR* C677T (rs1801133), *MTHFR* A1298C (rs1801131), *DHFR* 19bp (rs70991108), and *CBS* 68bp (rs876657421), have been analysed.

For the analysis of cytokine and methylation levels, a comparison was made between the cohort of patients with ASD and the control group of siblings. Conversely, for the intra-case analysis, the clinical characteristics of patients with ASD were utilized. The main clinical data considered included DSM-5 diagnostic criteria, ADOS scores, symptom severity, presence of OCD, intellectual disability based on IQ, and aggressive behaviour. Additionally, patients with ASD were stratified based on comorbidities such as epilepsy, airway infections, chronic gastrointestinal disorders (CGD), intestinal candida, gluten and lactose intolerances. Furthermore, a correlation analysis of *LINE-1* methylation levels between parents and children with or without ASD was conducted to identify correlations or differences in global DNA methylation levels between parents and children. Finally, experimental and clinical data underwent PCA and binary logistic regression to identify which biochemical, genetic, and epigenetic variables contribute to susceptibility to ASD.

Demographic Study of the Study Population

As a preliminary analysis, the demographic characteristics of the study population were identified, outlining the distribution between the male and female components of individuals with a confirmed diagnosis of ASD and the control group of SIBLINGS and related parents (table 3).

Table 3. Demographic Data of the Study Population

	ASD	SIBLINGS	Parents
Number of subjects	75	25	135
Sex n (%)			
Males	62 (82.7)	13 (52.0)	65 (48.1)
Females	13 (17.3)	12 (48.0)	70 (51.9)

In table 3, it can be observed that the ASD patient group comprised 62 male individuals (82.7%) and 13 female individuals (17.3%), totalling 75 ASD patients. In contrast, the SIBLINGS group consisted of 13 male individuals (52%) and 12 female individuals (48%), totalling 25 subjects. The parents included 65 fathers (48.1%) and 70 mothers (51.9%).

An important factor considered for a proper comparison of the study populations is age. The following table 4 shows the mean and median age in the ASD group, SIBLINGS group, and the Parents group. The analysis revealed that the distribution of individuals in the ASD and SIBLINGS groups ($p = 0.32$) is comparable for age. The minimum age of ASD patients was 3 years (yr), while the maximum age was 39 yr, with a median of 11 yr and a mean of 14.1 ± 7.95 yr. The SIBLINGS group had an age range of 3 to 28 yr, with a median of 15 yr and a mean of 15.3 ± 7.45 yr. The table 4 also shows the age of the examined groups stratified by gender where it is possible to observe that there are no statistically significant differences between the two sexes in both ASD and Siblings groups.

Table 4. Age of the Study Population

	ASD	SIBLINGS	ASD M	ASD F	Brothers	Sisters	Parents	Mothers	Fathers
Number Of Subjects	75	25	62	13	13	12	135	70	65
Age									
Minimum	3	3	3	6	6	3	30	30	33
25% Percentile	8	9	7.75	8	9.5	8.25	42	40	43
Median	**11**	**15**	**10**	**15**	**18**	**11.5**	**48**	**47**	**50**
75% Percentile	21	22	19.5	22	25	18.3	55	54	58
Maximum	39	28	39	23	28	23	68	65	68
Mean±SD	**14.1±7.95**	**15.3±7.45**	**13.8±8.22**	**15.4±6.63**	**17.9±7.92**	**12.5±5.99**	**48.7±9.1**	**47.3±8.64**	**50.2±9.4**

***P*value**	0.32	0.35	0.11	0.07
ASD M *vs* Brothers_ p-value	0.09			
ASD F *vs* Sisters_ p-value	0.35			

Clinical characterisations of participants with ASD

The intra-case analysis of the ASD group was carried out by exploiting the clinical characteristics of individuals diagnosed with ASD in order to investigate whether biochemical, genetic and epigenetic factors examined in this study may influence the degree of severity of ASD or discriminate patients based on symptoms or comorbidities. In fact, ASD individuals have been characterized based on the main clinical features and comorbidities. The main clinical data we considered were the results of the DSM-5 diagnostic manual, the ADOS diagnostic tool, the degree of severity of symptoms, the presence of OCD, intellectual disability based on the assessed IQ, and aggression based on the use of neuroleptic drugs. In this study we also considered the presence of comorbidities in patients because they can help to better understand the diversity between individuals with ASD and the severity of the phenotype. Comorbidities include epilepsy, airway infections, CGD, intestinal candida, and lactose and gluten intolerance. In the table 5, ASD individuals have been stratified for the different clinical characteristics identifying the number of subjects for the different subclasses and the percentage concerning the total number of individuals (n=75).

Table 5. Clinical characterisations of ASD groups

ASD individuals (n=75)			
Principal clinical data		**Comorbidities**	
DSM-5	**n (%)**	**Epilepsy**	**n (%)**
mild	7 (9.3)	NO	13 (17.3)
moderate	21 (28)	YES	62 (82.7)
severe	47 (62.7)	**Airway infections**	**n (%)**
ADOS	**n (%)**	never	20 (26.7)
mild	3 (4)	often	40 (53.3)
moderate	22 (29.3)	always	15 (20)
severe	50 (66.7)	**CGD**	**n (%)**
SYMPTOMS	**n (%)**	NO	18 (24)
no/minimal	24 (32)	YES	57 (76)
moderate	49 (65.3)	**Intestinal Candida**	**n (%)**
severe	2 (2.7)	never	23 (30.7)

OCD	n (%)
NO	33 (44)
YES	42 (56)
IQ	**n (%)**
normal	11 (14.7)
mild/moderate	40 (53)
severe	24 (32)
Aggressive behaviour	**n (%)**
NO	42 (56)
YES	33 (44)
often	29 (38.7)
always	23 (30.7)
Gluten Intolerance	**n (%)**
NO	26 (34.7)
YES	49 (65.3)
Lactose Intolerance	**n (%)**
NO	24 (32)
YES	51 (68)

The stratification of the ASD group based on clinical data such as DSM-5 and ADOS made it possible to identify three phenotypes: mild, moderate and severe. Patient characterization for DSM-5 identified a representative number for each of the three phenotypes, whereas stratification for ADOS found only 3 subjects with mild phenotype, so for intra-phenotype analysis, these individuals were considered together with the 22 patients with moderate phenotype and compared with the 50 severe ASD individuals. A similar strategy has been adopted for the analysis of patients stratified by the severity of the symptoms recorded the 2 individuals with severe phenotype identified were considered together with the 46 individuals with moderate symptoms and compared with the 24 ASD subjects with minimal or no symptoms.

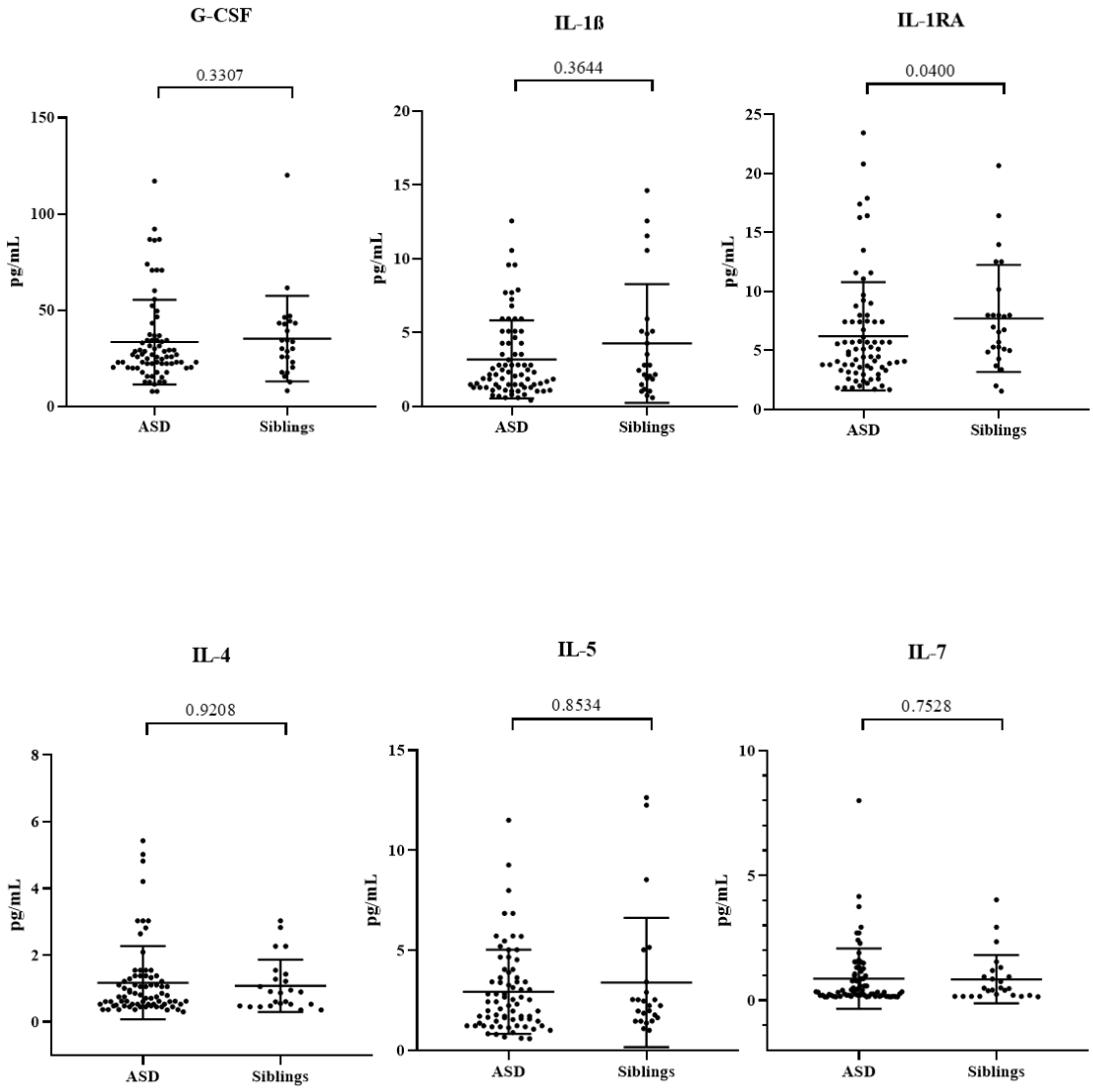
The subdivision for intellectual disability stratified patients with normal, mild/moderate, and severely impaired IQ. Stratifying for airway infections and intestinal candida, the ASD group was divided into three subclasses, patients who did not have comorbidity, patients who often suffered from it, and patients in whom comorbidity was always present. Finally, the stratification for the remaining clinical characteristics divided the individuals into the presence or absence of the clinical data.

Cytokine Analysis between ASD and SIBLINGS groups

The biochemical factors considered in the ASD study were plasma cytokines. As a starting sample, we used platelet-poor plasma and not platelet-rich plasma or serum, to avoid the uncontrolled release of cytokines by platelets and to avoid the coagulation process that would alter the biochemical analysis. In addition, the SIBLINGS group was used as a comparison group in the cytokine analyses because it is composed of individuals with a comparable age

to the ASD group (see "Demographic Study of the Study Population" paragraph); furthermore, being blood brothers, they share a good percentage of the genetic composition and are influenced by similar environmental factors.

Of the 20 cytokines analysed, only 11 were found to be detectable in the plasma samples analysed, namely G-CSF, IL-1β, IL-1RA, IL-4, IL-5, IL-7, IL-8, IL-10, IL-12(p40), MCP-1 and TNF-α. The comparison of the 11 cytokines between the ASD and SIBLINGS groups is shown in figure 13 and in table 6.



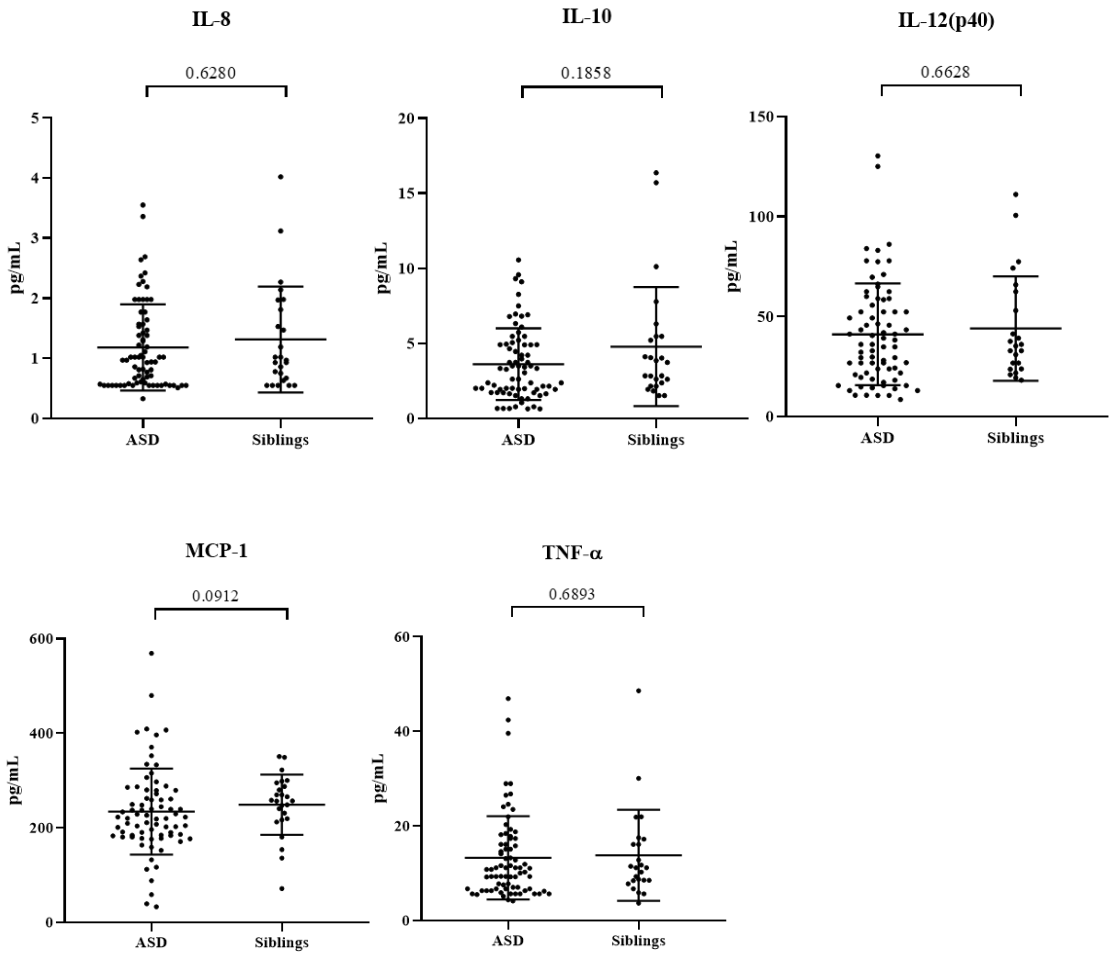


Figure 13. Cytokine levels of the ASD and SIBLINGS groups. The dot plots display cytokine levels expressed in pg/mL. Each graph shows the mean and SD, while the p-value between the analysed categories is reported at the top.

Table 6. Cytokine levels of ASD patients and SIBLINGS group

	Cytokines (pg/mL)	G-CSF	IL-1β	IL-1RA	IL-4	IL-5	IL-7	IL-8	IL-10	IL-12(p40)	MCP-1	TNF-α
ASD (75)	Minimum	7.84	0.41	1.67	0.3	0.59	0.15	0.33	0.65	8.52	32.98	4.14
	25% Percentile	20.34	1.28	3.31	0.53	1.415	0.21	0.57	1.835	21.59	183.7	6.71
	Median	**26.36**	**2.15**	**4.77**	**0.75**	**2.43**	**0.35**	**0.97**	**3.28**	**36.03**	**222.8**	**10.8**
	75% Percentile	35.02	4.375	7.43	1.33	3.76	1.17	1.57	4.94	56.38	279.2	17.34
	Maximum	117.1	12.56	23.45	5.43	11.5	8	3.55	10.56	130.3	569	46.9
	Mean±SD	**33.46 ±21.98**	**3.18 ±2.64**	**6.21 ±4.59**	**1.17 ±1.1**	**2.93 ±2.10**	**0.87 ±1.21**	**1.18 ±0.72**	**3.62 ±2.39**	**41.09 ±25.41**	**234.3 ±90.96**	**13.26 ±8.79**
SIBLINGS (25)	Minimum	8.24	0.59	1.55	0.36	1	0.15	0.55	1.52	18.19	71.76	3.68
	25% Percentile	21.02	1.57	4.93	0.492	1.51	0.185	0.65	2.27	23.84	218.3	8.438

Median	**31.86**	**2.62**	**6.75**	**0.885**	**2.24**	**0.48**	**0.97**	**3.74**	**35.31**	**256.8**	**11.17**
75% Percentile	43.29	5.09	9.075	1.393	3.29	1.075	1.89	5.475	62.45	291.6	16.94
Maximum	120.2	14.62	20.67	3.03	12.63	4.03	4.02	16.37	111.1	350.9	48.59
Mean±SD	**35.25 ±22.21**	**4.26 ±4.02**	**7.7± 4.54**	**1.08 ±0.78**	**3.4± 3.23**	**0.85 ±0.97**	**1.31 ±0.88**	**4.78 ±3.96**	**44.01 ±26.08**	**248.9 ±64.02**	**13.80 ±9.62**
***p*-value**	0.331	0.364	**0.040**	0.921	0.853	0.753	0.628	0.186	0.663	0.091	0.689

Statistically significant p-values ($p < 0.05$) are indicated in bold.

The analysis revealed that the plasma samples from ASD patients showed a statistically significant reduction in the cytokine IL-1RA compared to the control group ($p = 0.04$). The mean concentration of IL-1RA in the ASD group was 6.21 pg/mL, while the mean value in the SIBLINGS group was 7.7 pg/mL.

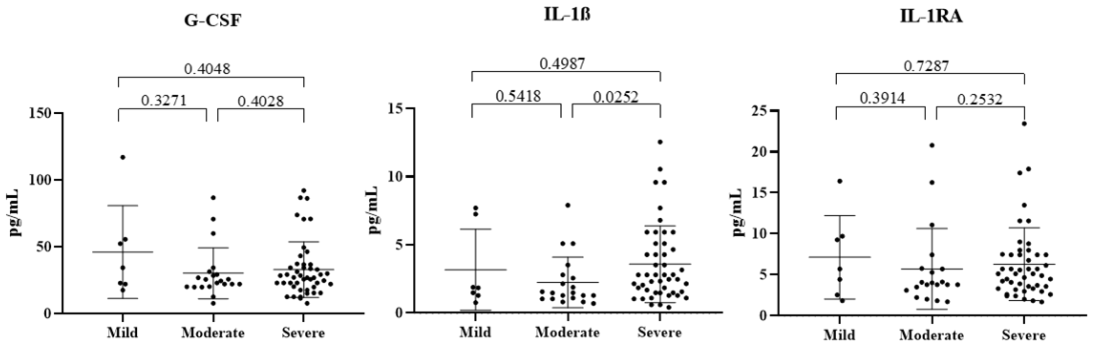
In addition, cytokine analysis also showed that chemokine G-CSF has lower levels in the ASD group than in the SIBLINGS group (median: 222.8 and 256.8 pg/mL respectively; $p = 0.33$).

Considering that males are more frequently affected by ASD, we compared cytokine levels by stratifying both the ASD group and the Siblings group by sex. This analysis did not show any significant results (data not shown).

Intra-case Cytokine Analysis stratified by Clinical Data

To gain insight into cytokines release in ASD patients, we conducted an intra-case analysis to identify potential changes in plasma cytokine concentrations based on the disorder severity. Previous studies show a link between immune dysfunction and behavioural traits of autism, so, with this analysis, we want to identify autistic subgroups with specific immunophenotypes. First, we performed a comparison between cytokines release and patients stratified by key clinical data such as DSM-5, ADOS, symptoms, OCD, intellectual disability (IQ), and aggression. A second analysis instead considered the comorbidities of the recruited individuals with ASD, since it was seen that they influence the course and severity of the disease. In this specific case, comorbidities such as CGD, airway infections, intestinal candida, epilepsy, and gluten and lactose intolerances were taken into consideration.

The initial analysis involved stratifying ASD patients based on the severity of the DSM-5 diagnostic criteria, categorizing the 75 patients into mild (n=7), moderate (n=21), and severe (n=47). The analysis, as shown in figure 14 and table 7, revealed that IL-1β levels were statistically higher in the severe phenotype compared to the moderate phenotype ($p = 0.025$), with cytokine concentrations of 3.58 pg/mL and 2.24 pg/mL, respectively. Another cytokine showing concentration variation was TNF-α, notably, the mild phenotype according to DSM-5 exhibited a statistically significant increase (19.5 pg/mL) compared to both the moderate phenotype (12.1 pg/mL; $p = 0.010$) and the severe phenotype (12.8 pg/mL; $p = 0.032$).



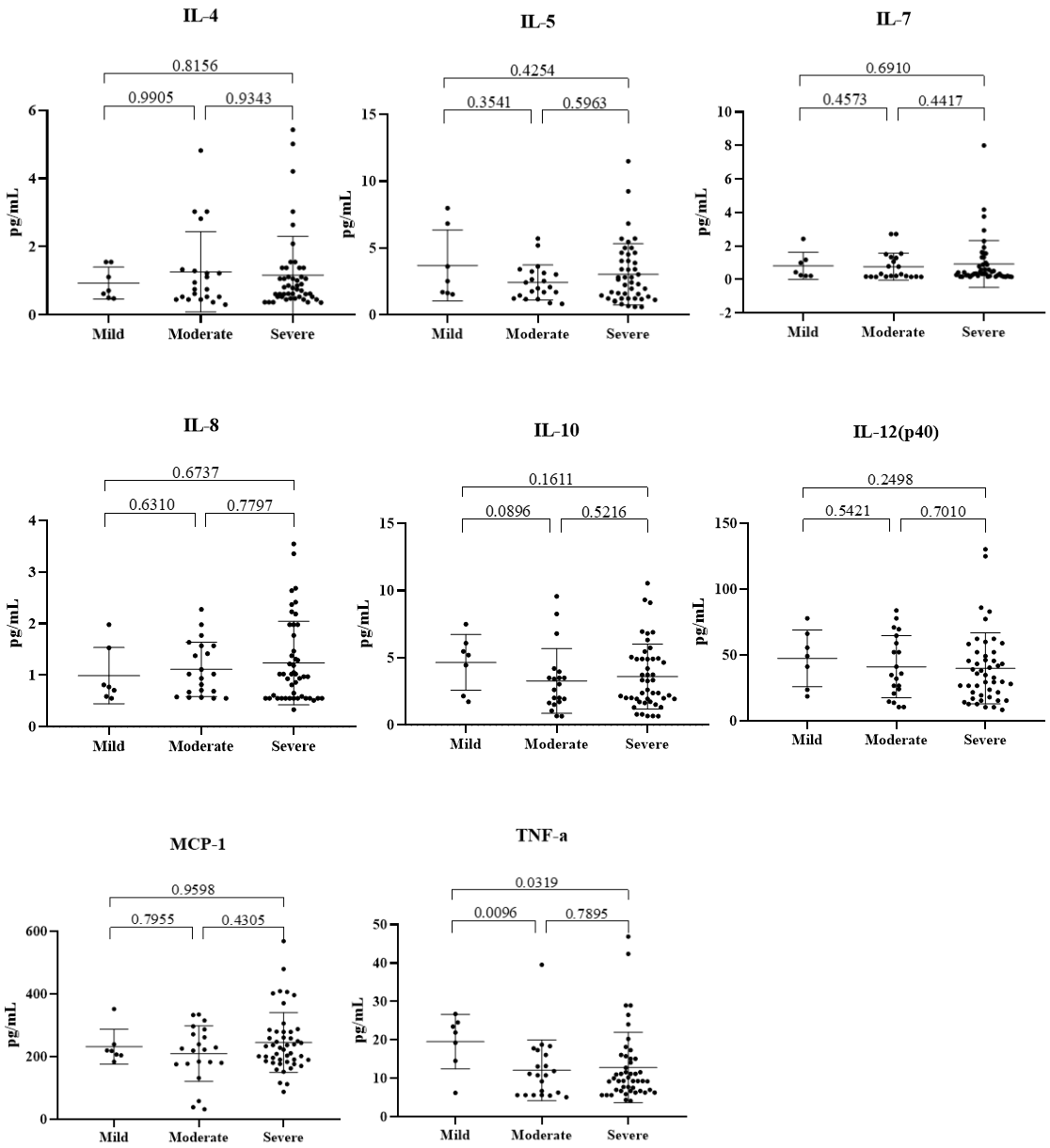


Figure 14. Cytokine levels of ASD patient stratified by DSM-5. The dot plots display cytokine levels expressed in pg/mL. Each graph shows the mean and SD, while the p-value between the analysed categories is reported at the top.

Table 7. Cytokine levels of ASD patient stratified by DSM-5

DSM-5	Cytokines (pg/mL)	G-CSF	IL-1β	IL-1RA	IL-4	IL-5	IL-7	IL-8	IL-10	IL-12(p40)	MCP-1	TNF-α
MILD (n=7)	Minimum	17.7	0.75	1.83	0.48	1.54	0.21	0.55	1.73	18.8	184	6.22
	25% Percentile	22.2	1.28	2.54	0.5	1.65	0.21	0.59	2.16	23.8	205	14.6
	Median	**34.4**	**1.84**	**5.7**	**0.71**	**2.53**	**0.43**	**0.77**	**5.21**	**49.3**	**219**	**22**

	75% Percentile	55.7	7.25	9.7	1.55	6.85	1.17	1.53	6.09	66.3	240	24.6
	Maximum	117	7.71	16.4	1.55	7.99	2.42	1.98	7.5	77.9	352	26.8
	Mean±SD	**46.1 ±34.7**	**3.17 ±2.97**	**7.13 ±5.1**	**0.93 ±0.47**	**3.7± 2.66**	**0.81 ±0.81**	**0.99 ±0.54**	**4.66 ±2.08**	**59.2 ±47.6**	**232 ±55.5**	**19.5 ±7.08**
MODERATE (n=21)	Minimum	7.84	0.69	1.7	0.3	0.83	0.15	0.55	0.65	10.7	33	5.12
	25% Percentile	20.3	1.05	3.2	0.49	1.35	0.17	0.625	1.67	21.8	177	5.64
	Median	**24.6**	**1.48**	**3.9**	**0.75**	**2.08**	**0.31**	**1.02**	**2.84**	**35.5**	**223**	**10.8**
	75% Percentile	30.4	2.74	5.75	1.31	3.2	1.31	1.57	3.85	63.4	279	16.7
	Maximum	86.8	7.9	20.8	4.82	5.72	2.71	2.28	9.58	84	335	39.6
	Mean±SD	**30.3 ±19.1**	**2.24 ±1.86**	**5.70 ±4.94**	**1.26 ±1.18**	**2.43 ±1.32**	**0.76 ±0.81**	**1.11 ±0.53**	**3.28 ±2.42**	**41.2 ±23.6**	**210 ±88.8**	**12.1 ±7.91**
SEVERE (n=47)	Minimum	7.84	0.41	1.67	0.36	0.59	0.15	0.33	0.65	8.52	88.4	4.14
	25% Percentile	20.2	1.48	3.31	0.53	1.23	0.21	0.55	1.89	19.6	186	6.71
	Median	**27.1**	**2.8**	**5.11**	**0.79**	**2.43**	**0.35**	**0.995**	**2.95**	**34.9**	**229**	**9.36**
	75% Percentile	36.8	5.09	7.43	1.38	4.29	0.99	1.82	4.93	52.3	279	15.1
	Maximum	92.1	12.6	23.5	5.43	11.5	8	3.55	10.6	130	569	46.9
	Mean±SD	**33.0 ±20.8**	**3.58 ±2.82**	**6.29 ±4.44**	**1.17 ±1.14**	**3.04 ±2.29**	**0.93 ±1.4**	**1.24 ±0.81**	**3.6± 2.43**	**40.1 ±27.0**	**246 ±95.3**	**12.8 ±9.16**
MILD *vs* MODERATE	***p*value**	0.3271	0.542	0.3914	0.991	0.354	0.457	0.631	0.090	0.542	0.796	**0.010**
MODERATE *vs*SEVERE		0.4028	**0.025**	0.2532	0.934	0.5963	0.442	0.780	0.522	0.701	0.431	0.790
MILD *vs* SEVERE		0.4048	0.499	0.7287	0.816	0.4254	0.691	0.674	0.161	0.250	0.960	**0.032**

Statistically significant p-values ($p < 0.05$) are indicated in bold.

The second clinical parameter considered for intra-case cytokine analysis was the ADOS diagnostic tool. Stratification based on ADOS categorized ASD patients into those with a mild/moderate phenotype and those with a severe phenotype (Figure 15 and Table 8). The analysis revealed that IL-1RA levels were higher in ASD patients with a severe phenotype

(6.66 pg/mL) compared to those with a mild/moderate phenotype (5.27 pg/mL; table 8); p = 0.059.

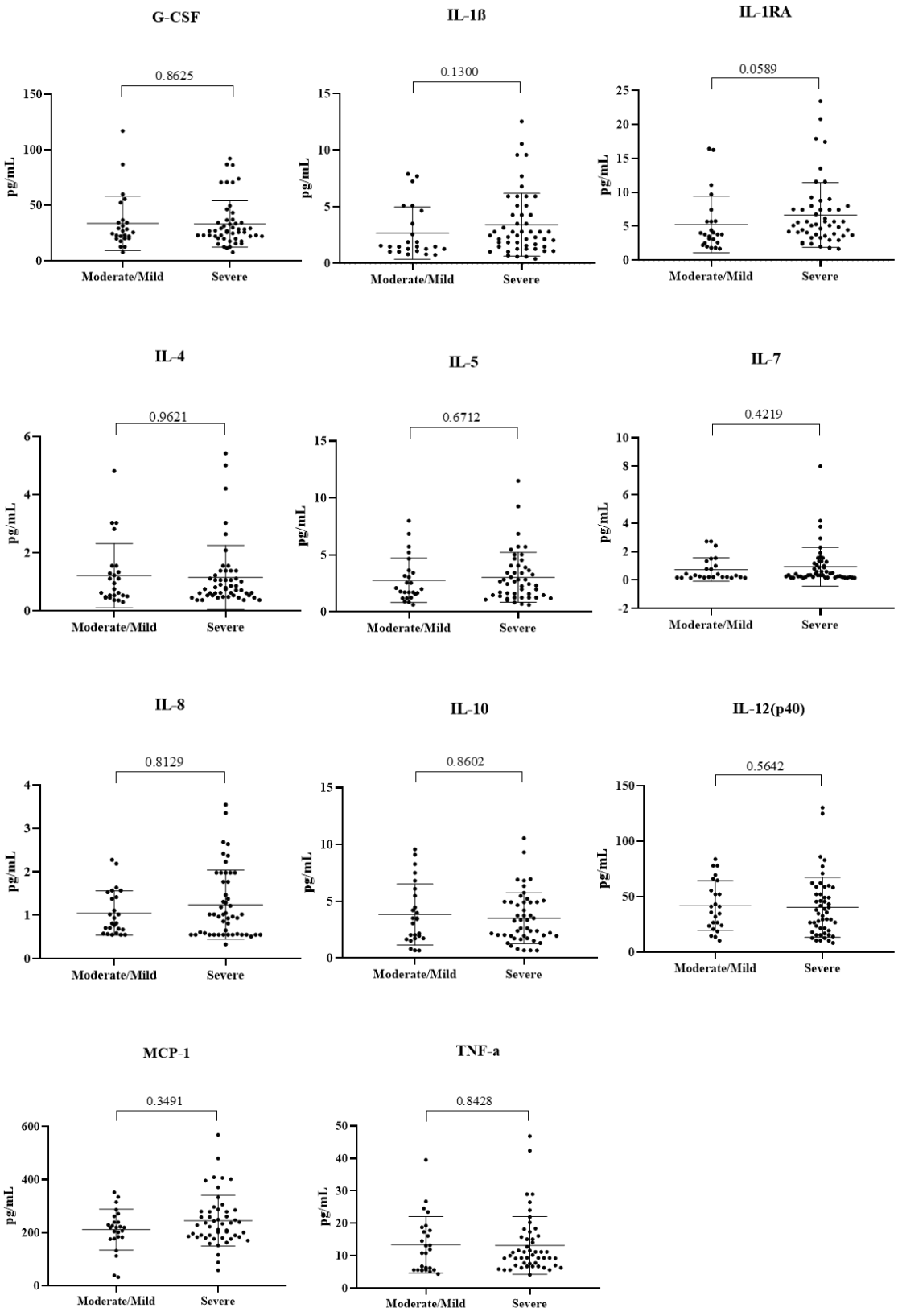


Figure 15. Cytokine levels of ASD patient stratified by ADOS. The dot plots display cytokine levels expressed in pg/mL. Each graph shows the mean and SD, while the p-value between the analysed categories is reported at the top.

Table 8. Cytokine levels of ASD patient stratified by ADOS

ADOS	Cytokines (pg/mL)	G-CSF	IL-1β	IL-1RA	IL-4	IL-5	IL-7	IL-8	IL-10	IL-12(p40)	MCP-1	TNF-α
MILD/MODERATE (n=25)	Minimum	7.84	0.75	1.7	0.3	0.61	0.15	0.55	0.65	10.6	33	4.38
	25% Percentile	20.3	1.14	2.55	0.49	1.39	0.185	0.63	1.73	23.9	180	5.64
	Median	**25.7**	**1.48**	**3.79**	**0.75**	**1.99**	**0.31**	**0.82**	**3.43**	**38.7**	**220**	**11.9**
	75% Percentile	35.6	4.38	5.75	1.44	3.53	1.16	1.48	5.79	62.6	251	18.3
	Maximum	117	7.9	16.4	4.82	7.99	2.71	2.28	9.58	84	352	39.6
	Mean±SD	**33.9 ±24.5**	**2.68 ±2.31**	**5.27 ±4.16**	**1.21 ±1.11**	**2.75 ±1.95**	**0.74 ±0.83**	**1.05 ±0.51**	**3.83 ±2.69**	**42.1 ±22.3**	**212 ±76.9**	**13.4 ±8.69**
SEVERE (n=50)	Minimum	7.84	0.41	1.67	0.37	0.59	0.15	0.33	0.65	8.52	58.5	4.14
	25% Percentile	21.3	1.48	3.66	0.53	1.39	0.21	0.55	1.94	19.2	184	7.03
	Median	**26.9**	**2.47**	**5.2**	**0.77**	2.55	**0.385**	**1.02**	2.95	**35.5**	**231**	**10.1**
	75% Percentile	35.6	4.48	7.61	1.26	4	1.2	1.88	4.92	53.9	282	15.9
	Maximum	92.1	12.6	23.5	5.43	11.5	8	3.55	10.6	130	569	46.9
	Mean±SD	**33.3 ±20.8**	**3.41 ±2.77**	**6.66 ±4.76**	**1.15 ±1.11**	**3.02 ±2.20**	**0.94 ±1.36**	**1.25 ±0.8**	**3.50 ±2.24**	**40.6 ±27.0**	**246 ±96.0**	**13.2 ±8.93**
MILD/MODERATE *vs* SEVERE	***p*value**	0.863	0.130	**0.059**	0.962	0.671	0.422	0.813	0.860	0.564	0.349	0.843

Statistically significant p-values ($p < 0.05$) are indicated in bold.

The ASD group was then stratified using the degree of clinically experienced symptoms. From the subdivision, patients were grouped into individuals with no or minimal symptoms and individuals with moderate and severe symptoms of ASD.

In figure 16 and table 9 are shown the results of the analysis of plasma cytokine levels in patients with ASD stratified by symptoms. The analysis shows a slight increase in IL-1β in moderate/severe patients (3.38 pg/mL) compared to the group with minimal or no symptoms

(2.73 pg/mL); p = 0.17. No statistically significant results were observed between the two groups analysed.

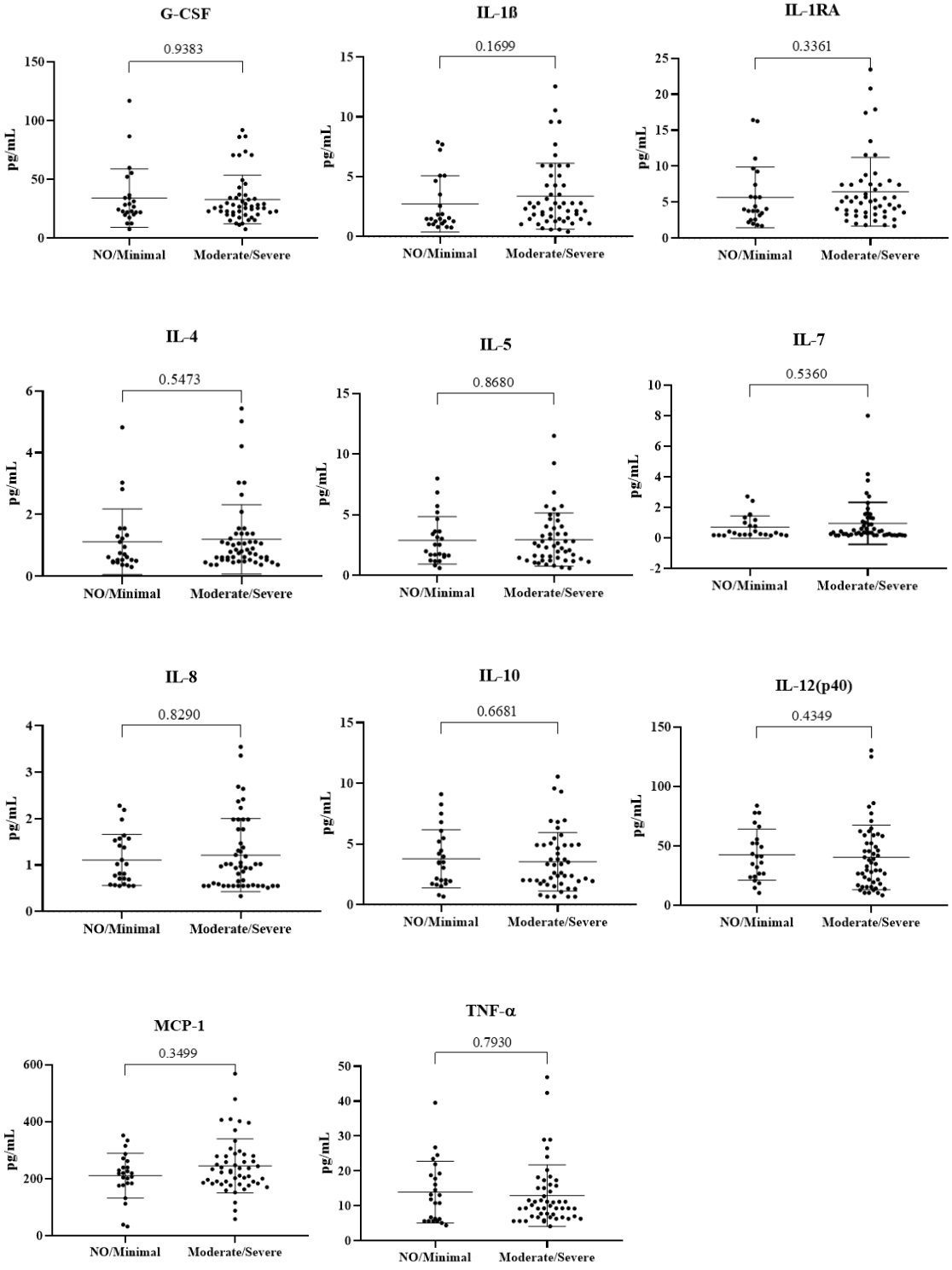


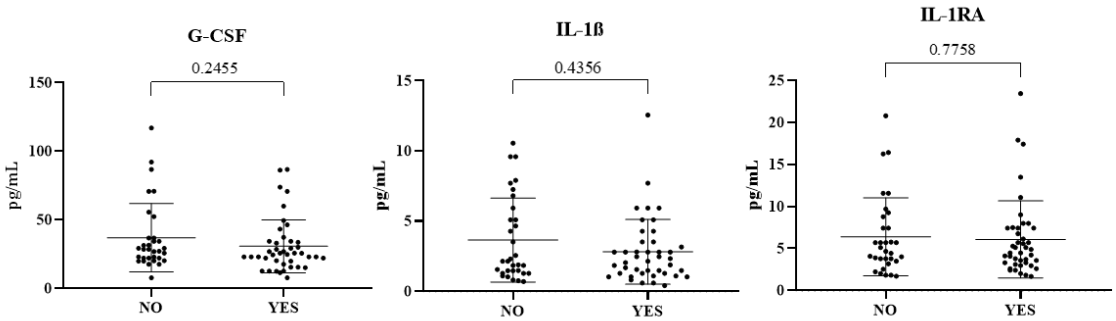
Figure 16. Cytokine levels of ASD patient stratified by Symptoms. The dot plots display cytokine levels expressed in pg/mL. Each graph shows the mean and SD, while the p-value between the analysed categories is reported at the top.

Table 9. Cytokine levels of ASD patient stratified by Symptoms

Symptoms	Cytokines (pg/mL)	G-CSF	IL-1β	IL-1RA	IL-4	IL-5	IL-7	IL-8	IL-10	IL-12(p40)	MCP-1	TNF-α
NO/MINIMAL (n=24)	Minimum	7.84	0.75	1.7	0.3	0.61	0.15	0.55	0.67	10.6	33	4.38
	25% Percentile	20.8	1.09	2.59	0.485	1.57	0.203	0.615	1.78	24.3	179	5.79
	Median	**25.7**	**1.48**	**4**	**0.665**	**2.26**	**0.325**	**0.92**	**3.47**	**41.3**	**219**	**12.5**
	75% Percentile	36.2	4.66	7.43	1.32	3.63	1.13	1.56	5.41	55.7	257	19.1
	Maximum	117	7.9	16.4	4.82	7.99	2.71	2.28	9.11	84	352	39.6
	Mean±SD	**34.3 ±24.9**	**2.73 ±2.35**	**5.68± 4.23**	**1.12 ±1.06**	**2.89 ±1.95**	**0.70 ±0.73**	**1.11 ±0.55**	**3.78 ±2.40**	**42.7 ±21.5**	**211 ±78.5**	**13.9 ±8.85**
MODERATE/SEVERE (n=51)	Minimum	7.84	0.41	1.67	0.37	0.59	0.15	0.33	0.65	8.52	58.5	4.14
	25% Percentile	20.3	1.47	3.58	0.53	1.3	0.21	0.55	1.84	18.2	184	7.03
	Median	**26.4**	**2.47**	**5.11**	**0.81**	**2.43**	**0.35**	**0.97**	**2.62**	**34.9**	**229**	**10**
	75% Percentile	35	4.28	7.43	1.38	3.97	1.28	1.77	4.92	58.5	280	15.8
	Maximum	92.1	12.6	23.5	5.43	11.5	8.00	3.55	10.6	130	569	46.9
	Mean±SD	**33.1 ±20.7**	**3.38 ±2.76**	**6.45± 4.77**	**1.20 ±1.12**	**2.95 ±2.20**	**0.95 ±1.37**	**1.21 ±0.79**	**2.54 ±2.41**	**40.4 ±27.2**	**245 ±95.1**	**12.9 ±8.84**
	***p*value**	0.938	0.170	0.336	0.547	0.868	0.536	0.829	0.668	0.435	0.350	0.793

Statistically significant p-values ($p < 0.05$) are indicated in bold.

For the cytokine analysis, another parameter we considered was the presence of OCD in ASD patients (figure 17 and table 10). ASD group stratification revealed that patients with OCD had lower TNF-α concentrations compared to ASD patients without OCD, at 11.4 pg/ml and 15.6 pg/ml, respectively ($p = 0.053$).



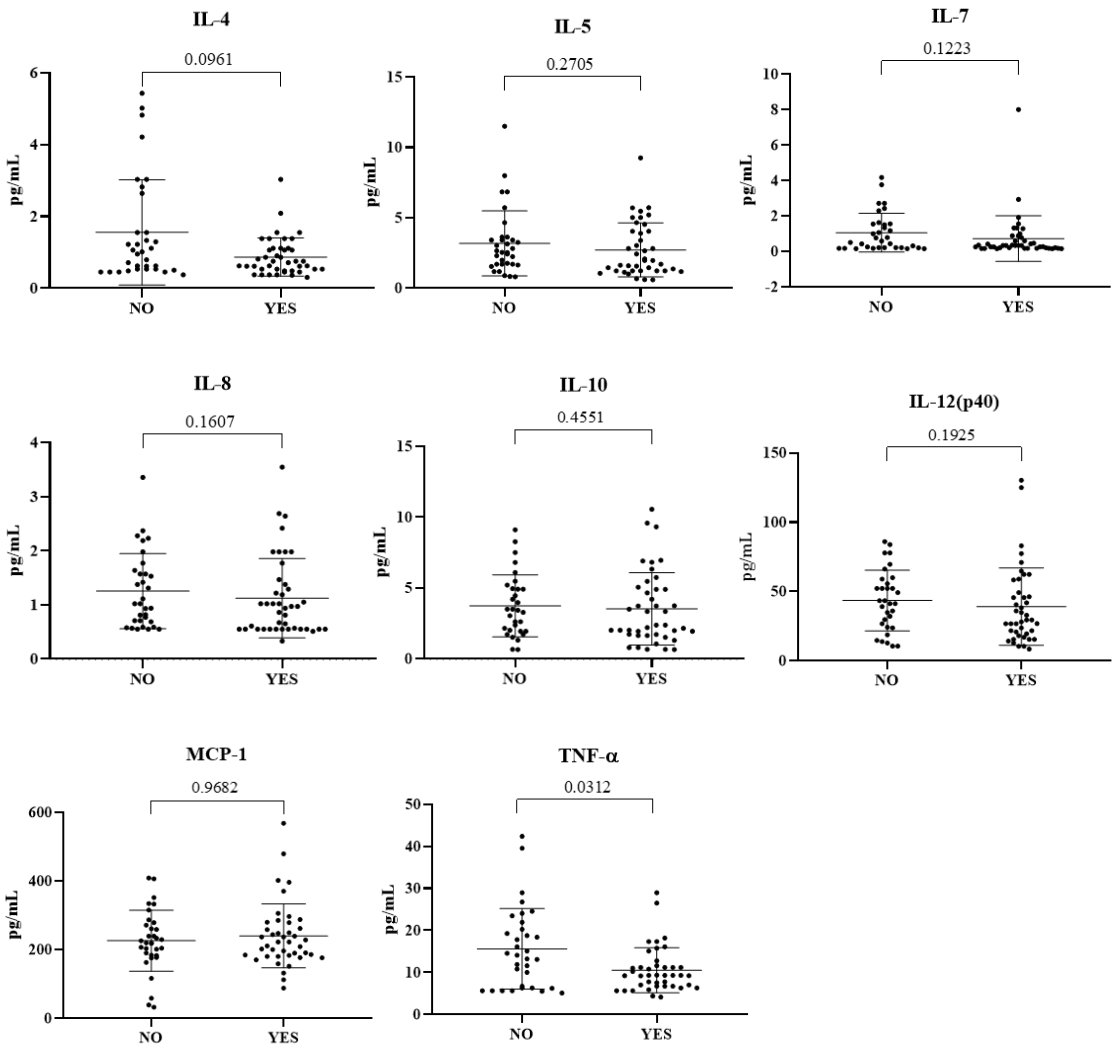


Figure 17. Cytokine levels of ASD patient stratified by OCD. The dot plots display cytokine levels expressed in pg/mL. Each graph shows the mean and SD, while the p-value between the analysed categories is reported at the top.

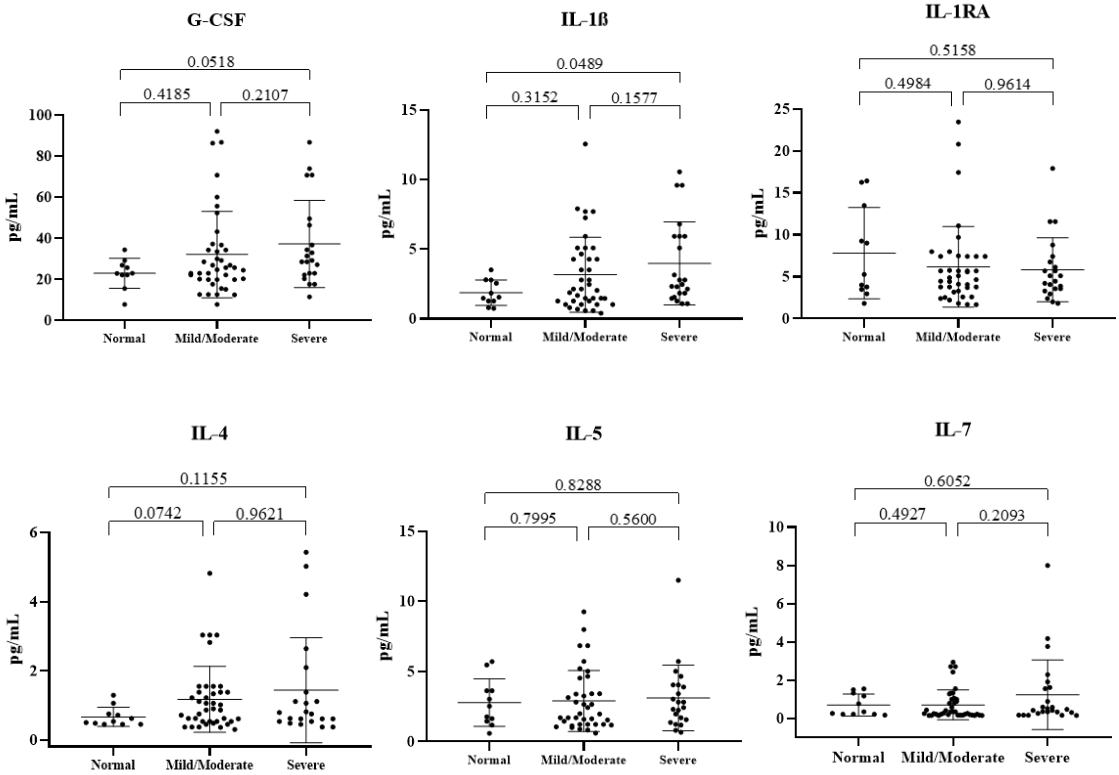
Table 10. Cytokine levels of ASD patient stratified by OCD

OCD	Cytokines (pg/mL)	G-CSF	IL-1β	IL-1RA	IL-4	IL-5	IL-7	IL-8	IL-10	IL-12(p40)	MCP-1	TNF-α
NO (n=33)	Minimum	7.84	0.69	1.7	0.37	0.8	0.15	0.55	0.65	10.7	33	5.12
	25% Percentile	22.2	1.33	3.57	0.53	1.71	0.21	0.695	1.94	24.9	184	6.33
	Median	**28.6**	**2.15**	**4.89**	**1.01**	**2.54**	**0.58**	**1.02**	**3.43**	**42.4**	**220**	**14.1**
	75% Percentile	36.8	5.72	8.44	2.1	3.53	1.55	1.62	4.96	58.2	276	21.1
	Maximum	117	10.6	20.8	5.43	11.5	4.17	3.36	9.11	86.1	409	42.4
	Mean±SD	**37.0 ±24.9**	**3.65 ±2.99**	**6.38 ±4.65**	**1.56 ±1.47**	**3.18 ±2.31**	**1.06± 1.09**	**1.25 ±0.69**	**3.74 ±2.19**	**43.6± 21.9**	**227 ±88.8**	**15.6 ±9.61**
	Minimum	7.84	0.41	1.67	0.3	0.59	0.15	0.33	0.65	8.52	88.4	4.14

YES(n=42)	25% Percentile	19.4	1.28	3.26	0.518	1.23	0.19	0.55	1.7	19.2	183	6.71
	Median	**25.3**	**2.24**	**4.68**	**0.73**	**1.97**	**0.34**	**0.955**	**2.38**	**29.5**	**223**	**9.31**
	75% Percentile	34.4	3.52	7.43	1.11	4.04	0.80	1.4	4.95	51.6	280	11.9
	Maximum	86.8	12.6	23.5	3.03	9.26	8.00	3.55	10.6	130	569	46.9
	Mean±SD	**30.8 ±19.3**	**2.81 ±2.30**	**6.08 ±4.60**	**0.87 ±0.53**	**2.72 ±1.92**	**0.725 ±1.29**	**1.12 ±0.73**	**3.53 ±2.55**	**39.2± 27.9**	**240 ±93.2**	**11.4 ±7.71**
	***P* value**	0.2455	0.4356	0.7758	0.0961	0.2705	0.122	0.1607	0.455	0.1925	0.9682	**0.053**

Statistically significant p-values ($p < 0.05$) are indicated in bold.

To assess whether the cytokine release was different in patients with intellectual disabilities, we stratified the cohort of ASD considering the alteration of the IQ. This stratification allowed us to identify three groups: individuals with normal IQ, individuals with mild/moderate IQ alteration, and subjects with severe alterations. As shown in figure 18 and table 11, cytokine analysis showed that the phenotype of severe intellectual disability had statistically higher levels of G-CSF, IL-1β, and MCP-1 than patients with a normal IQ, with $p = 0.051$, $p = 0.049$ and $p = 0.015$, respectively.



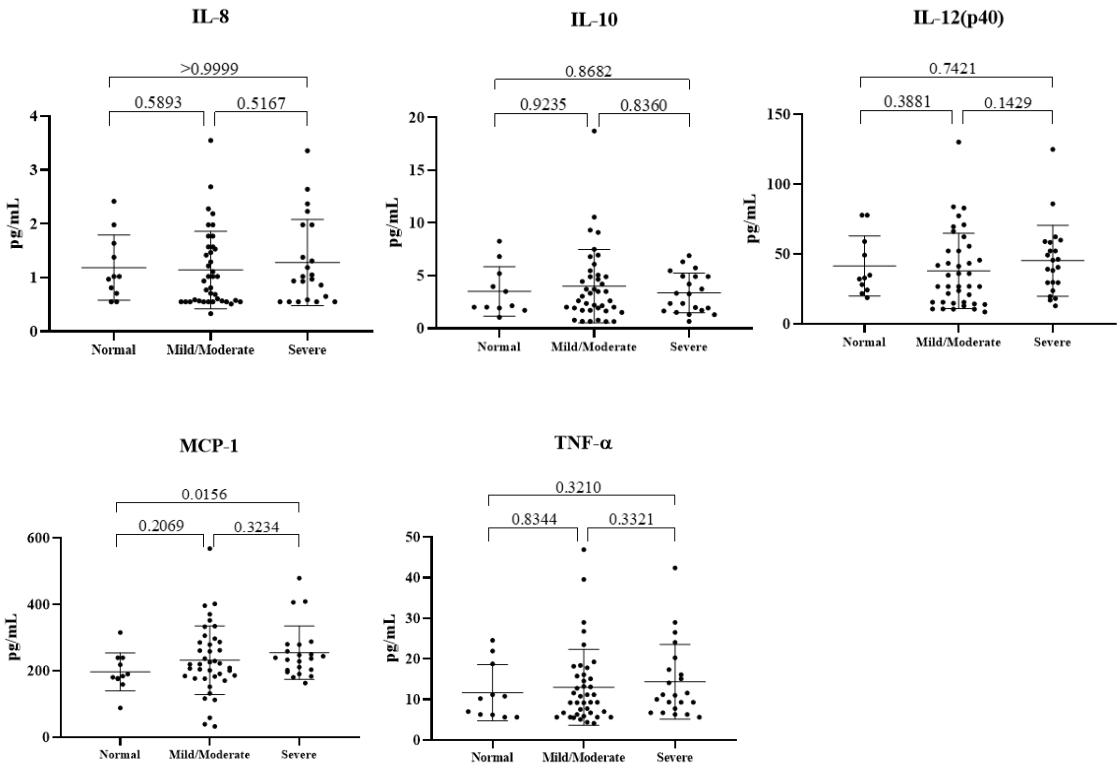


Figure 18. Cytokine levels of ASD patient stratified by IQ. The dot plots display cytokine levels expressed in pg/mL. Each graph shows the mean and SD, while the p-value between the analysed categories is reported at the top.

Table 11. Cytokine levels of ASD patient stratified by IQ

IQ	Cytokines (pg/mL)	G-CSF	IL-1β	IL-1RA	IL-4	IL-5	IL-7	IL-8	IL-10	IL-12(p40)	MCP-1	TNF-α
Normal (n=11)	Minimum	7.84	0.75	1.83	0.45	0.59	0.17	0.55	1.05	18.8	88.4	5.64
	25% Percentile	20.6	1.28	3.49	0.45	1.46	0.21	0.71	1.94	24.3	176	6.22
	Median	**23**	**1.55**	**5.28**	**0.53**	**2.53**	**0.34**	**1.02**	**2.16**	**32.9**	**184**	**10.2**
	75% Percentile	27.5	2.8	13.5	0.75	3.63	1.32	1.64	5.21	59.1	240	18.8
	Maximum	34.4	3.52	16.4	1.29	5.7	1.54	2.42	8.27	77.9	316	24.6
	Mean±SD	**23.0 ±7.30**	**1.88 ±0.91**	**7.80 ±5.45**	**0.66 ±0.28**	**2.79 ±1.69**	**0.70 ±0.57**	**1.19 ±0.61**	**3.52 ±2.34**	**41.5 ±21.5**	**197 ±57.2**	**11.7 ±6.91**
Mild/Moderate (n=40)	Minimum	7.84	0.41	1.67	0.3	0.61	0.15	0.33	0.65	8.52	33	4.14
	25% Percentile	20	1.28	3.31	0.53	1.23	0.178	0.55	1.78	15.5	179	6.43
	Median	**25.3**	**2.15**	**4.88**	**0.88**	**1.98**	**0.305**	**0.94**	**3.2**	**27**	**220**	**10.1**

	75% Percentile	36.2	4.66	7.43	1.38	3.7	0.99	1.57	5.02	52.3	287	16
	Maximum	92.1	12.6	23.5	4.82	9.26	2.94	3.55	18.7	130	569	46.9
	Mean±SD	**32.2 ±21.0**	**3.17 ±2.69**	**6.17 ±4.81**	**1.17 ±0.95**	**2.90 ±2.16**	**0.70 ±0.78**	**1.14 ±0.72**	**4.01 ±3.48**	**38.0 ±27.0**	**232 ±103**	**13.0 ±9.32**
SEVERE (n=24)	Minimum	11.6	1.09	1.83	0.37	0.67	0.15	0.55	0.67	13	163	5.64
	25% Percentile	22.6	1.77	3.51	0.53	1.52	0.27	0.58	1.69	28.1	201	7.49
	Median	**29.3**	**2.64**	**4.79**	**0.77**	**2.63**	**0.44**	**0.995**	**3.28**	**43.1**	**239**	**11.1**
	75% Percentile	48	5.93	6.92	1.56	4.04	1.60	1.98	4.94	58.6	279	18.1
	Maximum	86.8	10.6	17.9	5.43	11.5	8.00	3.36	6.91	125	480	42.4
	Mean±SD	**37.3 ±4.63**	**3.98 ±2.98**	**5.82 ±3.80**	**1.44 ±1.52**	**3.11 ±2.34**	**1.23 ±1.82**	**1.28 ±0.8**	**3.37 ±1.87**	**45.3 ±25.4**	**255 ±80.3**	**14.4 ±9.20**
NORMAL *vs* MILD/MODERATE	***p*value**	0.4185	0.3152	0.4984	0.0742	0.7995	0.4927	0.5893	0.9235	0.3881	0.2069	0.8344
MILD/MODERATE *vs*SEVERE		0.2107	0.1577	0.9614	0.9621	0.56	0.209	0.5167	0.836	0.1429	0.3234	0.3321
NORMAL *vs* SEVERE		**0.0518**	**0.0489**	0.5158	0.1155	0.8288	0.605	ns	0.8682	0.7421	**0.0156**	0.321

Statistically significant p-values ($p < 0.05$) are indicated in bold.

The last of the main clinical features considered in the intra-case analysis was the presence of aggressive behaviour in patients. The presence of aggression was assessed based on the use of neuroleptic drugs, in fact the ASD group was stratified into patients who took antipsychotic drugs or not. Intra-case analysis (shown in figure 19 and table 12) revealed that IL-12(p40) and TNF-α levels were statistically lower in ASD individuals with aggressive phenotype compared to the others. Specifically, IL-12(p40) levels were 34.7 pg/ml and 46.2 pg/ml ($p = 0.0261$) in the presence and absence of neuroleptic drug use, respectively. In contrast, TNF-α was 11.1 pg/ml in patients using antipsychotic drugs and 14.9 pg/ml in patients not taking them ($p = 0.048$).

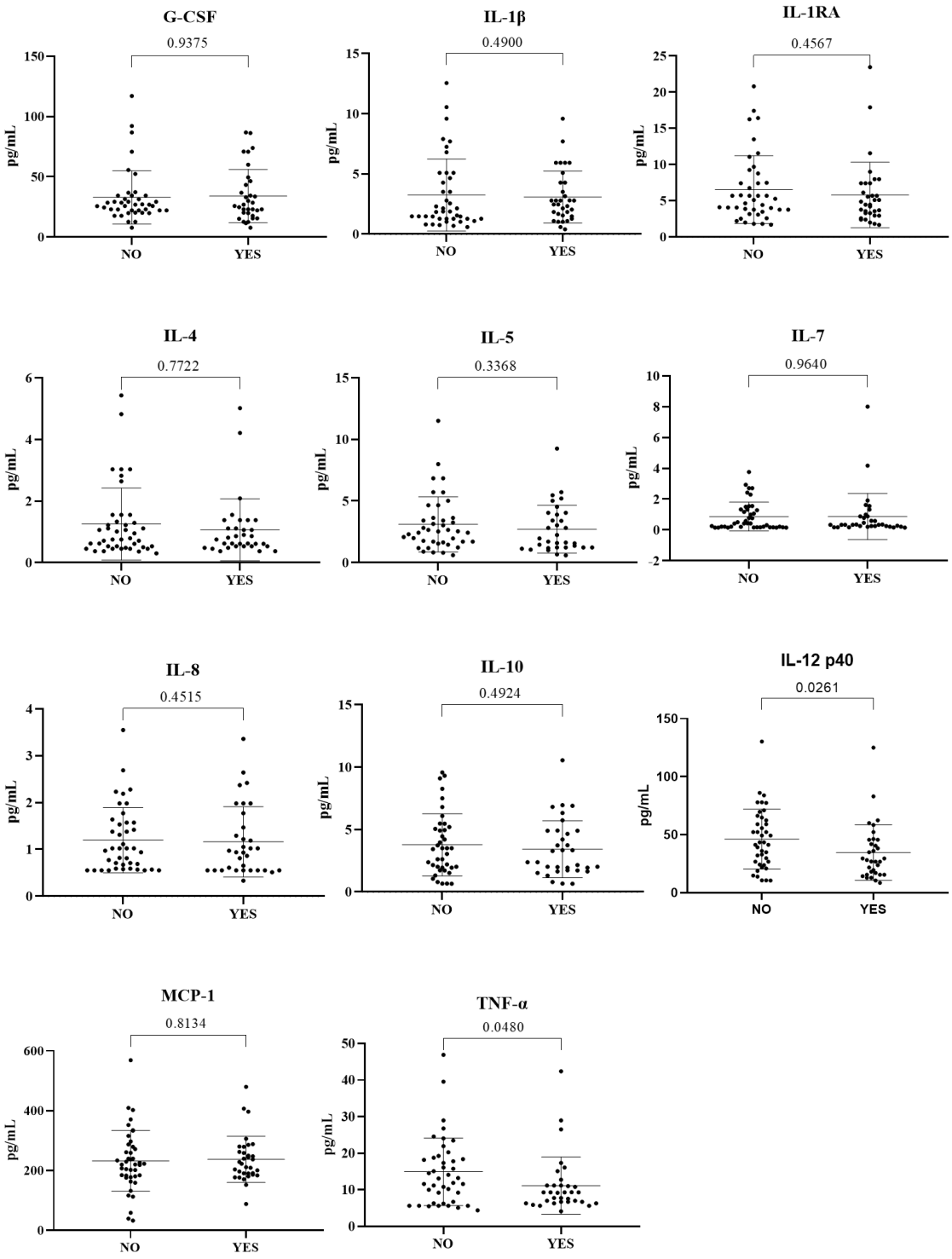


Figure 19. Cytokine levels of ASD patient stratified by Aggressive Behaviour. The dot plots display cytokine levels expressed in pg/mL. Each graph shows the mean and SD, while the p-value between the analysed categories is reported at the top.

Table 12. Cytokine levels of ASD patient stratified by use of Aggressive Behaviour

Aggressive Behaviour	Cytokines (pg/mL)	G-CSF	IL-1β	IL-1RA	IL-4	IL-5	IL-7	IL-8	IL-10	IL-12(p40)	MCP-1	TNF-α
NO (n=42)	Minimum	7.84	0.59	1.7	0.3	0.61	0.15	0.55	0.65	10.6	33	4.38
	25% Percentile	22.2	1.28	3.64	0.495	1.68	0.17	0.59	1.94	25.5	180	6.62
	Median	**26.9**	**1.88**	**5.11**	**0.77**	**2.53**	**0.42**	**1.02**	**3.47**	**43.4**	**222**	**13.2**
	75% Percentile	34.4	4.88	8.14	1.39	3.63	1.32	1.57	5.17	63.6	281	18.9
	Maximum	117	12.6	20.8	5.43	11.5	3.76	3.55	9.58	130	569	46.9
	Mean ±SD	**32.9 ±22.1**	**3.25 ±2.99**	**6.54 ±4.67**	**1.25 ±1.17**	**3.10 ±2.23**	**0.87 ±0.87**	**1.19 ±0.67**	**3.78 ±2.49**	**46.2± 25.7**	**232 ±101**	**14.9 ±9.23**
YES(n=33)	Minimum	7.84	0.41	1.67	0.37	0.59	0.15	0.33	0.65	8.52	88.4	4.14
	25% Percentile	18.8	1.52	3.14	0.53	1.23	0.23	0.55	1.73	16.3	188	6.71
	Median	**25.8**	**2.47**	**4.47**	**0.75**	**1.84**	**0.34**	**0.94**	**2.38**	**28.1**	**223**	**9.31**
	75% Percentile	44.9	4.28	7.43	1.25	4	0.88	1.62	4.91	45.7	271	11.2
	Maximum	86.8	9.59	23.5	5.02	9.26	8	3.36	10.6	125	480	42.4
	Mean ±SD	**34.1 ±22.1**	**3.08 ±2.16**	**5.81 ±4.52**	**1.07 ±1.0**	**2.71 ±1.94**	**0.87 ±1.5**	**1.16 ±0.75**	**3.42 ±2.28**	**34.7± 24.0**	**237 ±77.0**	**11.1 ±7.84**
	***p*value**	0.938	0.49	0.4567	0.7722	0.3368	0.964	0.4515	0.4924	**0.0261**	0.8134	**0.048**

Statistically significant p-values ($p < 0.05$) are indicated in bold.

The second part of the analysis conducted on the inflammatory system of the ASD group focused attention on the patients' comorbidities and how these can influence the course of the disease. The objective was to identify possible differences in cytokine release among more severe clinical manifestations and to recognize specific phenotype markers for various comorbidity in individual with ASD.

The first comorbidity we analysed was the presence of epilepsy events in ASD subjects and the results are shown in figure 20 and table 13.

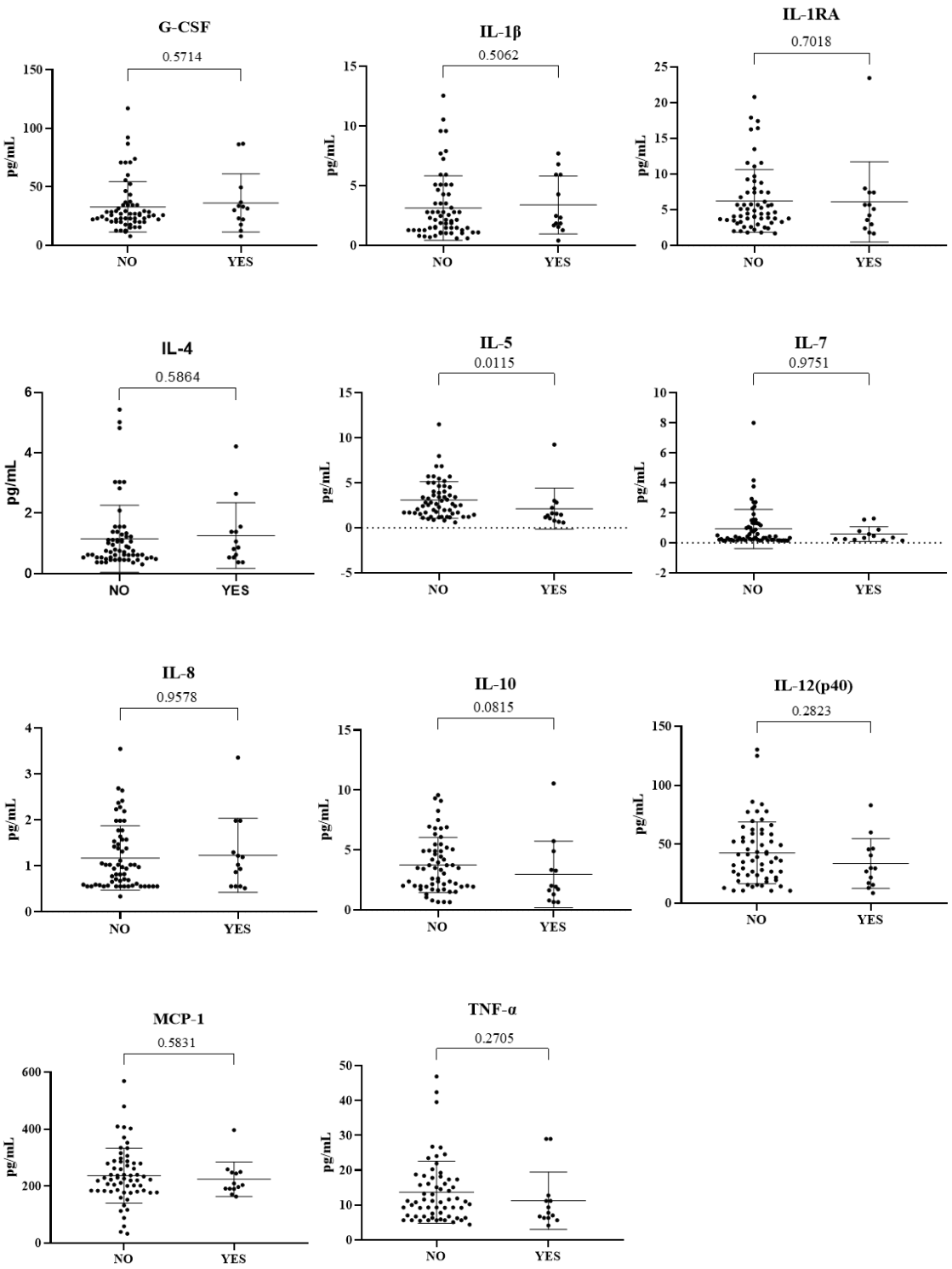


Figure 20. Cytokine levels of ASD patient stratified by use of Epilepsy. The dot plots display cytokine levels expressed in pg/mL. Each graph shows the mean and SD, while the p-value between the analysed categories is reported at the top.

Table 13. Cytokine levels of ASD patient stratified by use of Epilepsy

Epilepsy	Cytokines (pg/mL)	G-CSF	IL-1β	IL-1RA	IL-4	IL-5	IL-7	IL-8	IL-10	IL-12(p40)	MCP-1	TNF-α
NO (n=60)	Minimum	7.84	0.59	1.7	0.3	0.61	0.15	0.33	0.65	10.6	33	4.38
	25% Percentile	20.3	1.28	3.4	0.523	1.61	0.2	0.575	1.95	22.8	183	6.71
	Median	**25.8**	**2.15**	**4.66**	**0.75**	**2.6**	**0.345**	**0.97**	**3.47**	**38.3**	**225**	**11.1**
	75% Percentile	34.4	4.28	7.46	1.24	4.04	1.29	1.57	5.04	58.8	282	17.5
	Maximum	117	12.6	20.8	5.43	11.5	8	3.55	9.58	130	569	46.9
	Mean±SD	**32.9 ±21.5**	**3.13 ±2.70**	**6.23 ±4.39**	**1.15 ±1.11**	**3.10 ±2.04**	**0.93 ±1.30**	**1.17 ±0.7**	**3.76 ±2.3**	**42.7± 26.1**	**236 ±96.4**	**13.7 ±8.9**
YES(n=13)	Minimum	7.84	0.41	1.67	0.37	0.59	0.15	0.51	0.65	8.52	163	4.14
	25% Percentile	20	1.62	2.69	0.53	0.93	0.23	0.55	1.05	16.3	190	6.33
	Median	**31.5**	**2.33**	**5.11**	**0.86**	**1.46**	**0.35**	**1.02**	**1.94**	**29.5**	**204**	**7.75**
	75% Percentile	43.2	5.93	7.43	1.47	2.53	0.83	1.64	4.13	46	249	12
	Maximum	86.8	7.71	23.5	4.21	9.26	1.62	3.36	10.6	83.1	397	29
	Mean±SD	**36.2 ±24.8**	**3.39 ±2.42**	**6.11 ±5.63**	**1.25 ±1.09**	**2.13 ±2.27**	**0.58 ±0.5**	**1.23 ±0.81**	**2.97 ±2.78**	**33.7± 21.2**	**224 ±60.5**	**11.3 ±8.24**
	***p*value**	0.5714	0.5062	0.7018	0.5864	**0.0115**	0.9751	0.9578	0.0815	0.2823	0.5831	0.2705

Statistically significant p-values ($p < 0.05$) are indicated in bold.

The results showed that IL-5 and IL-10 levels were lower in ASD patients with epilepsy (IL-5 = 2.13 pg/mL; IL-10 = 2.97 pg/mL) compared to those do not suffer from it (IL-5 = 3.10 pg/mL; IL-10 = 3.76 pg/mL), although only IL-5 presents a statistically significant difference ($p = 0.011$).

Another comorbidity found in patients with ASD is airway infections. Figure 21 and table 14 show the results of the analysis of cytokines levels stratified by the presence and frequency of respiratory tract infections.

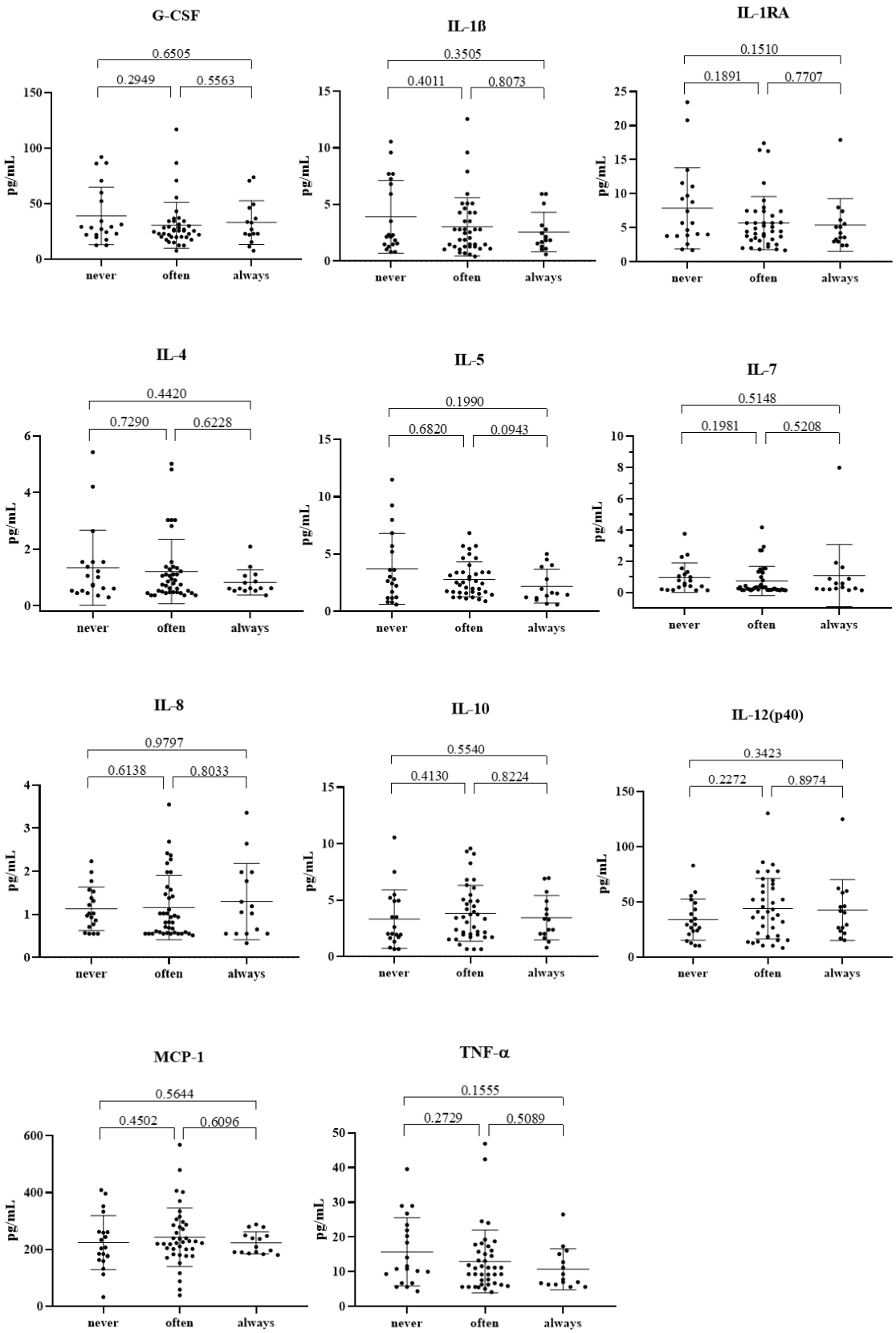


Figure 21. Cytokine levels of ASD patient stratified by Airway Infections. The dot plots display cytokine levels expressed in pg/mL. Each graph shows the mean and SD, while the p-value between the analysed categories is reported at the top.

Table 14. Cytokine levels of ASD patient stratified by Airway Infections

Airway infections	Cytokines (pg/mL)	G-CSF	IL-1β	IL-1RA	IL-4	IL-5	IL-7	IL-8	IL-10	IL-12(p40)	MCP-1	TNF-α
never (n=20)	Minimum	12.7	0.8	1.7	0.3	0.61	0.15	0.55	0.67	10.6	33	4.38
	25% Percentile	22.2	1.48	3.82	0.53	1.19	0.243	0.71	1.65	21.7	167	7.37
	Median	**29**	**2.23**	**5.7**	**0.88**	**2.74**	**0.67**	**1.02**	**2.02**	**29.7**	**206**	**11.2**
	75% Percentile	58.2	7.14	10.7	1.55	5.58	1.28	1.53	4.96	47.9	262	23.1
	Maximum	92.1	10.6	23.5	5.43	11.5	3.76	2.23	10.6	83.1	409	39.6
	Mean±SD	**39.2 ±25.9**	**3.91 ±3.22**	**7.86 ±5.95**	**1.35 ±1.33**	**3.72 ±3.09**	**0.96 ±0.94**	**1.13 ±0.5**	**3.32 ±2.59**	**34.1± 18.6**	**224 ±94.7**	**15.7 ±9.82**
often (n=40)	Minimum	7.84	0.41	1.67	0.37	0.89	0.15	0.51	0.65	8.52	39.5	4.14
	25% Percentile	20.2	1.28	3.17	0.48	1.64	0.178	0.573	1.94	18.8	188	6.43
	Median	**25.8**	**2.47**	**4.47**	**0.825**	**2.48**	**0.32**	**0.88**	**3.43**	**41.3**	**225**	**11.1**
	75% Percentile	34.4	4.28	7.43	1.32	3.42	1.21	1.55	5.06	64.8	287	16
	Maximum	117	12.6	17.4	5.02	6.85	4.17	3.55	9.58	130	569	46.9
	Mean±SD	**30.6 ±20.6**	**3.03 ±2.57**	**5.68 ±3.91**	**1.21 ±1.14**	**2.8± 1.52**	**0.75 ±0.94**	**1.16 ±0.74**	**3.83 ±2.48**	**44.0± 27.4**	**243 ±103**	**13.0 ±9.03**
always (n=15)	Minimum	7.84	0.59	2.4	0.37	0.59	0.15	0.33	0.79	15.5	181	5.64
	25% Percentile	22.2	1.28	2.97	0.53	1.18	0.21	0.55	2.02	23.8	190	6.33
	Median	**26.9**	**1.84**	**4.22**	**0.62**	**1.57**	**0.34**	**1.05**	**3.28**	**40.5**	**209**	**7.75**
	75% Percentile	46.5	3.16	6.13	1.06	3.89	0.88	1.98	4.91	58.5	250	15.1
	Maximum	74	5.93	17.9	2.09	5.02	8	3.36	6.96	125	288	26.5
	Mean±SD	**33.2 ±19.7**	**2.56 ±1.74**	**5.38 ±3.88**	**0.82 ±0.44**	**2.2± 1.47**	**1.09 ±1.99**	**1.30 ±0.88**	**3.44 ±1.96**	**42.8± 27.5**	**223 ±38.9**	**10.7 ±5.91**
never *vs* often	***p*value**	0.2949	0.4011	0.1891	0.729	0.682	0.1981	0.6138	0.413	0.2272	0.4502	0.2729
often *vs* always		0.5563	0.8073	0.7707	0.6228	0.0943	0.5208	0.8033	0.8224	0.8974	0.6096	0.5089
never *vs* always		0.6505	0.3505	0.151	0.442	0.199	0.5148	0.9797	0.554	0.3423	0.5644	0.1555

Statistically significant p-values ($p < 0.05$) are indicated in bold.

The analysis showed no statistically significant differences in cytokine levels based on the frequency of airway infections.

One of the main comorbidities found in ASD patients is CGD. Several studies have demonstrated the influence of the gut microbiome on the brain-gut axis, i.e. that the enteric system can influence the neuronal activity of the brain. A good percentage of ASD patients suffer from CGD and therefore we wanted to analyse the possible alterations in the systemic cytokine release in the ASD group. The results of the analysis are shown in figure 22 and table 15.

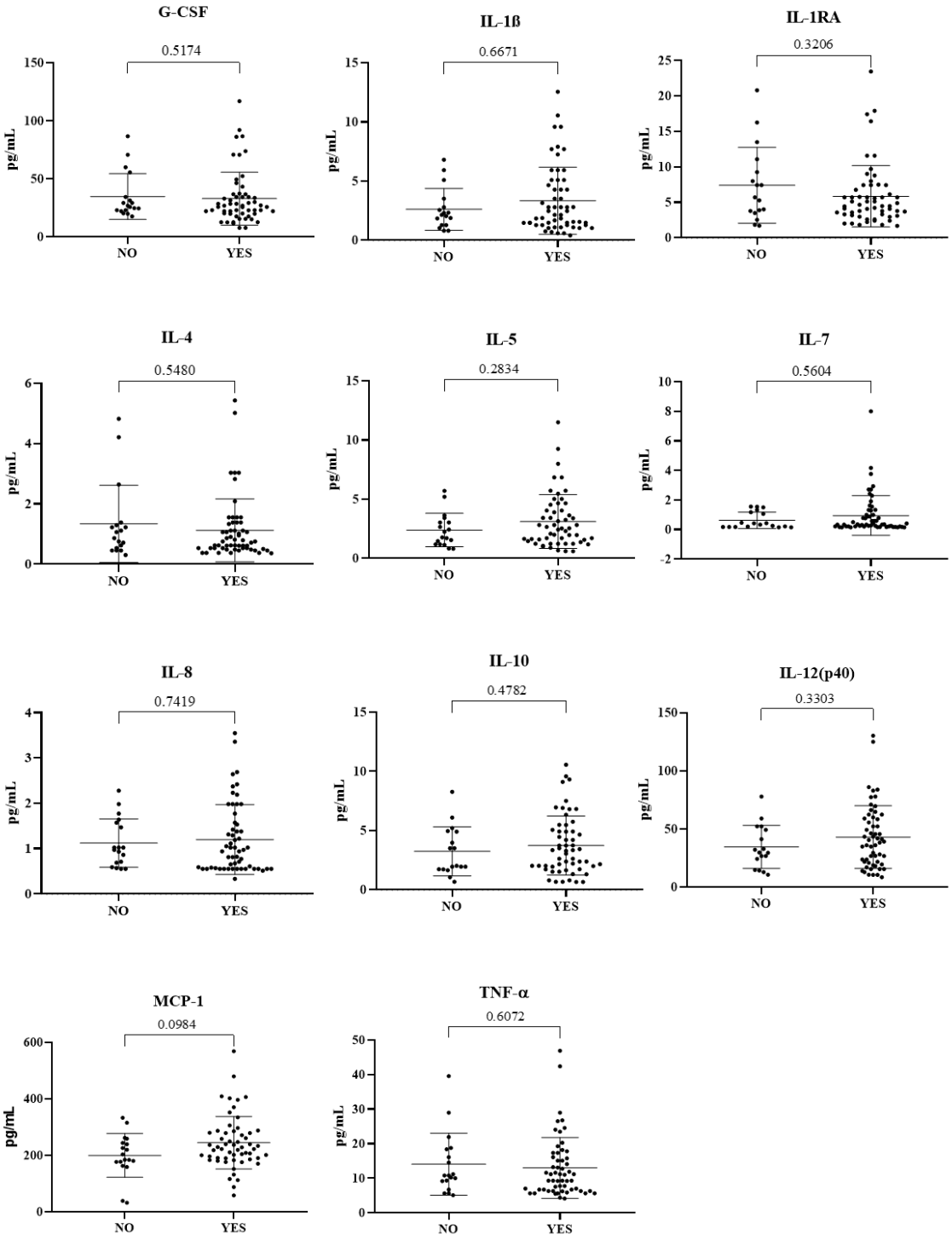


Figure 22. Cytokine levels of ASD patient stratified by CGD. The dot plots display cytokine levels expressed in pg/mL. Each graph shows the mean and SD, while the p-value between the analysed categories is reported at the top.

Table 15. Cytokine levels of ASD patient stratified by CGD

CGD	Cytokines (pg/mL)	G-CSF	IL-1β	IL-1RA	IL-4	IL-5	IL-7	IL-8	IL-10	IL-12(p40)	MCP-1	TNF-α
NO (n=18)	Minimum	17.7	0.8	1.7	0.3	0.8	0.15	0.55	0.67	10.7	33	5.12
	25% Percentile	22.8	1.28	3.64	0.51	1.18	0.17	0.665	1.73	19.6	174	8.58
	Median	26.4	2.15	5.7	0.96	2.01	0.38	0.995	2.02	29.8	194	10.8
	75% Percentile	39.8	3.16	10.2	1.31	3.13	1.21	1.59	4.94	50.8	248	18.5
	Maximum	86.8	6.81	20.8	4.82	5.7	1.55	2.28	8.27	77.9	333	39.6
	Mean±SD	**34.8± 19.8**	**2.62± 1.77**	**7.41± 5.36**	**1.34± 1.28**	**2.39± 1.42**	**0.93± 0.55**	**1.12± 0.53**	**3.24± 2.07**	**34.6± 18.5**	**200± 77.4**	**14.1± 8.95**
YES(n=57)	Minimum	7.84	0.41	1.67	0.36	0.59	0.15	0.33	0.65	8.52	58.5	4.14
	25% Percentile	20	1.28	3.24	0.53	1.46	0.21	0.55	1.95	21.4	190	6.52
	Median	26.4	2.15	4.47	0.75	2.53	0.35	0.955	3.31	39.2	229	11
	75% Percentile	36.2	4.88	7.09	1.36	4.04	1.14	1.56	5.03	59.5	283	16.7
	Maximum	117	12.6	23.5	5.43	11.5	8	3.55	10.6	130	569	46.9
	Mean±SD	**33.0± 22.8**	**3.34± 2.84**	**5.85± 4.32**	**1.12± 1.04**	**3.11± 2.27**	**0.95± 1.34**	**1.20± 0.77**	**3.73± 2.48**	**43.0± 27.0**	**245± 92.9**	**13.0± 8.81**
	***p*value**	0.5174	0.6671	0.3206	0.548	0.2834	0.5604	0.7419	0.4782	0.3303	0.0984	0.6072

Statistically significant p-values ($p < 0.05$) are indicated in bold.

Comparison of cytokine levels between ASD subjects with and without CGD showed no statistically significant results. We can in fact state that among the 11 cytokines analysed, only MCP-1 levels were slightly increased in the ASD subgroup suffering from CGD (245 pg/mL) compared to that without CGD (200 pg/mL); $p = 0.098$.

A CGD-related comorbidity is intestinal infection due to candida, which is why the ASD group has been stratified by the presence and frequency of candida infection events. Figure 23 and table 16 show the results of the analysis.

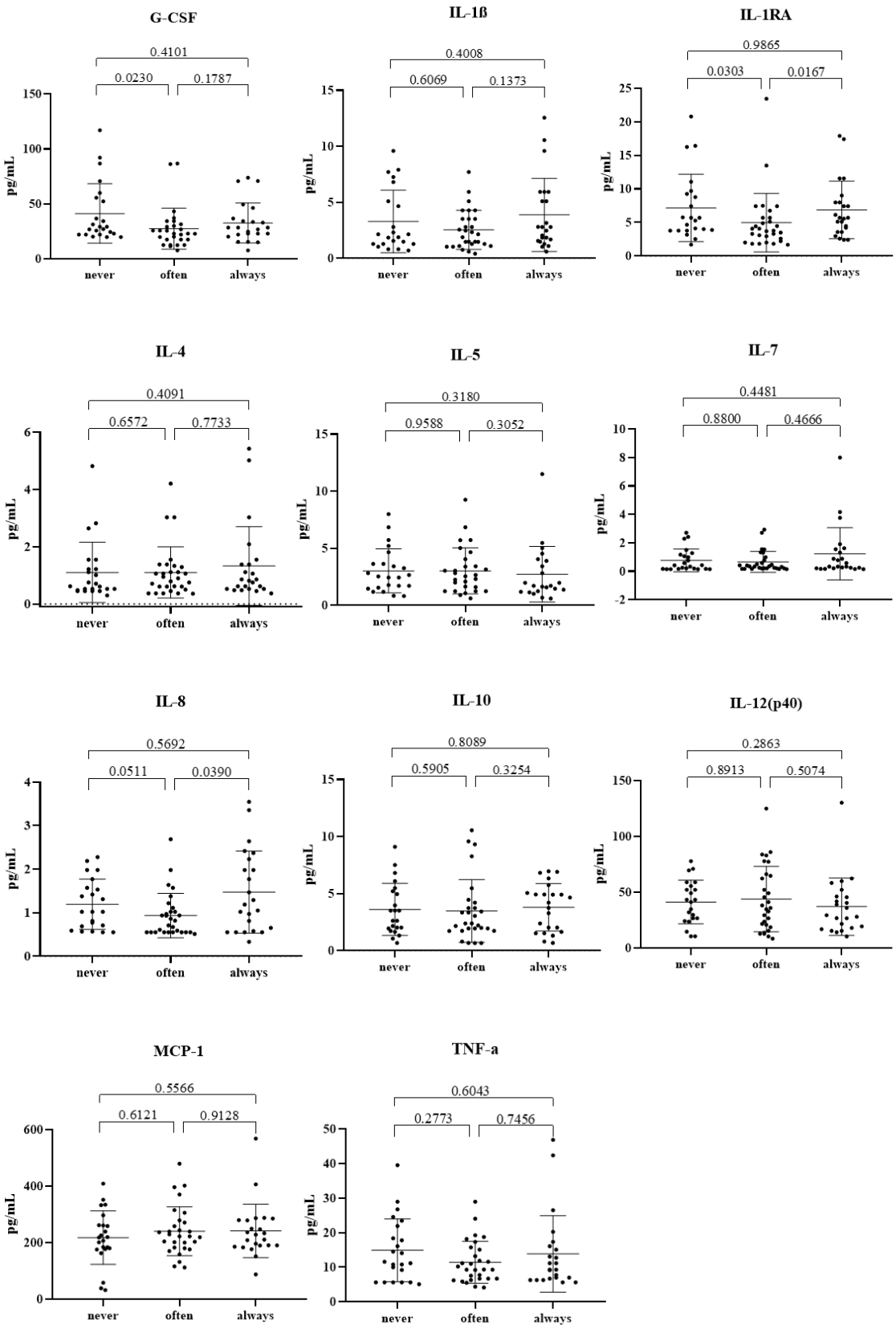


Figure 23. Cytokine levels of ASD patient stratified by Intestinal Candida. The dot plots display cytokine levels expressed in pg/mL. Each graph shows the mean and SD, while the p-value between the analysed categories is reported at the top.

Table 16. Cytokine levels of ASD patient stratified by Intestinal Candida

Intestinal Candida	Cytokines (pg/mL)	G-CSF	IL-1β	IL-1RA	IL-4	IL-5	IL-7	IL-8	IL-10	IL-12(p40)	MCP-1	TNF-α
never (n=23)	Minimum	20	0.69	1.7	0.3	0.8	0.15	0.55	0.67	10.7	33	5.12
	25% Percentile	22.2	1.28	3.79	0.48	1.54	0.17	0.665	1.89	26.2	178	5.64
	Median	**29.3**	**2.02**	**5.49**	**0.62**	**2.53**	**0.42**	**1.02**	**3.06**	**42.4**	**219**	**11.6**
	75% Percentile	55.7	5.52	9.36	1.22	3.63	1.17	1.62	5.28	56.5	262	22
	Maximum	117	9.59	20.8	4.82	7.99	2.71	2.28	9.11	77.9	409	39.6
	Mean±SD	**41.3 ±27.1**	**3.29 ±2.8**	**7.17 ±5.03**	**1.1± 1.05**	**3.02 ±1.93**	**0.77 ±0.79**	**1.19 ±0.58**	**3.61 ±2.28**	**41.3± 19.5**	**218± 94.3**	**14.9 ±9.11**
often (n=29)	Minimum	7.84	0.41	1.67	0.36	0.61	0.15	0.51	0.65	8.52	113	4.14
	25% Percentile	17.7	1.19	2.41	0.555	1.32	0.21	0.55	1.78	19.9	182	6.71
	Median	**23**	**2.14**	**3.79**	**0.9**	**2.6**	**0.35**	**0.82**	**2.38**	**36**	**223**	**9.36**
	75% Percentile	31.1	3.52	5.7	1.31	3.89	0.885	1.07	4.09	65.6	276	15.4
	Maximum	86.8	7.71	23.5	4.21	9.26	2.94	2.69	10.6	125	480	29
	Mean±SD	**27.6 ±18.7**	**2.54 ±1.75**	**4.97 ±4.34**	**1.1± 0.89**	**3.02 ±2.02**	**0.66 ±0.74**	**0.94 ±0.51**	**3.48 ±2.76**	**43.9± 29.3**	**241± 86.7**	**11.4 ±6.08**
always (n=23)	Minimum	7.84	0.59	2.4	0.37	0.59	0.15	0.33	0.67	10.6	88.4	5.64
	25% Percentile	22.2	1.55	3.58	0.53	1.22	0.21	0.58	1.65	18.2	190	6.71
	Median	**28.6**	**2.8**	**5.7**	**0.81**	**1.71**	**0.34**	**1.19**	**4.21**	**29.5**	**229**	**9.31**
	75% Percentile	36.8	5.93	7.98	1.38	3.93	1.55	2.23	5.06	46.4	280	16.1
	Maximum	74	12.6	17.9	5.43	11.5	8	3.55	6.96	130	569	46.9
	Mean±SD	**32.7 ±18.2**	**3.88 ±3.27**	**6.86 ±4.29**	**1.33 ±1.38**	**2.72 ±2.44**	**1.23 ±1.84**	**1.47 ±0.94**	**3.79 ±2.08**	**37.3± 25.7**	**242± 94.8**	**13.9 ±11.1**
never *vs* often	***p*value**	**0.023**	0.6069	**0.0303**	0.6572	0.9588	0.88	**0.0511**	0.5905	0.8913	0.6121	0.2773
often *vs* always		0.1787	0.1373	**0.0167**	0.7733	0.3052	0.4666	**0.039**	0.3254	0.5074	0.9128	0.7456
never *vs* always		0.4101	0.4008	0.9865	0.4091	0.318	0.4481	0.5692	0.8089	0.2863	0.5566	0.6043

Statistically significant p-values ($p < 0.05$) are indicated in bold.

Analysis of cytokines release in the ASD group stratified by intestinal candida infection showed that patients who often suffered from this infection had statistically lower levels of G-CSF, IL-1RA and IL-8 compared to the ASD subgroup without intestinal candida ($p = 0.023$, $p = 0.03$ and $p = 0.05$ respectively) and to that of ASD subjects who have always

suffered from intestinal candida. Although in the latter case, only the two cytokines IL-1RA and IL-8 showed a statistically significant variation ($p = 0.017$ and $p = 0.039$, respectively).

Two intolerances widely spread in the population and in patients with ASD have been considered comorbidities: lactose and gluten intolerance. Figure 24 and Table 17 show the only cytokines whose levels were found to be lower in ASD patients suffering from lactose and gluten intolerance.

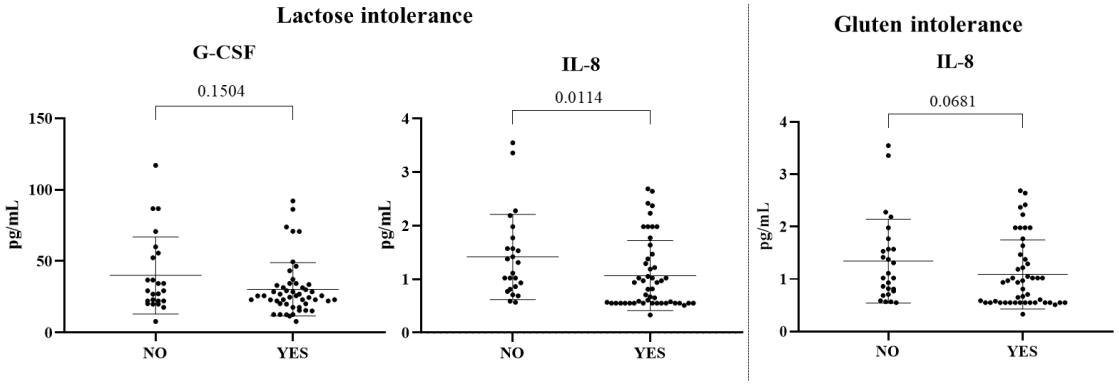


Figure 24. Cytokine levels of ASD patient stratified by Lactose and Gluten Intolerance. The dot plots display cytokine levels expressed in pg/mL. Each graph shows the mean and SD, while the p-value between the analysed categories is reported at the top.

Table 17. Cytokine levels of ASD patient stratified by Lactose and Gluten Intolerance

Intolerance	Cytokines (pg/mL)	G-CSF (Lactose)	IL-8 (Lactose)	IL-8 (Gluten)
NO (n=24)	Minimum	7.84	0.57	0.55
	25% Percentile	22.2	0.823	0.755
	Median	29.3	1.21	1.07
	75% Percentile	54.9	1.72	1.62
	Maximum	117	3.55	3.55
	Mean±SD	40.0±26.9	1.42±0.79	1.34±0.8
YES(n=51)	Minimum	7.84	0.33	0.33
	25% Percentile	20.1	0.55	0.55
	Median	25.8	0.815	0.94
	75% Percentile	33.2	1.4	1.45
	Maximum	92.1	2.69	2.69
	Mean±SD	30.3±18.7	1.07±0.65	1.09±0.66
	***p*value**	0.1504	**0.0114**	0.0681

Statistically significant p-values ($p < 0.05$) are indicated in bold.

ASD subgroups with lactose and gluten intolerance showed reduced IL-8 levels in individuals with intolerances. This reduction was statistically significant when lactose intolerance was considered ($p = 0.011$). This group of patients also shows a decreasing trend in G-CSF levels.

DNA Methylation Analysis between ASD and SIBLINGS and Genotypic Stratification

Global DNA methylation analysis was conducted using the percentage of methylation of CpG islands in the *LINE-1* promoter sequence. A comparison of *LINE-1* methylation was performed between ASD patients and the SIBLINGS group because, as highlighted in the analysis of demographic data, siblings were of a comparable age with patients, which is an indispensable requirement to comparing the epigenetic changes such as methylation which could vary depending on the age of the subjects. Figure 26 and table 19 report the mean methylation of the 4 CpG islands in the *LINE-1* promoter in the ASD and SIBLINGS groups.

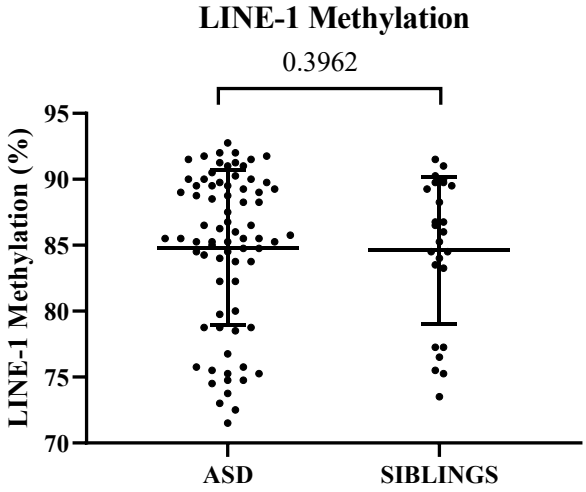


Figure 26. *LINE-1* methylation levels of ASD patient and SIBLINGS groups. The dot plots display *LINE-1* methylation expressed in %. Each graph shows the mean and SD, while the p-value between the analysed categories is reported at the top.

Table 19. *LINE-1* methylation levels of ASD patient and SIBLINGS groups

	ASD	**SIBLINGS**
Number of Subjects	75	25
***LINE-1*: Methylation of 4 CpG (%)**		
Minimum	71.5	73.5
25% Percentile	80	80.25
Median	**85.5**	**86.0**
75% Percentile	89.5	89.63
Maximum	92.75	91.5
Mean±SD	**84.81±5.87**	**84.62±5.58**
***p*-value**	0.396	

The analysis revealed no statistically significant difference between the ASD and SIBLINGS groups (p = 0.40). Comparison of *LINE-1* methylation levels was also performed in affected and unaffected individuals after stratification by sex. This analysis also did not produce statistically significant results (data not shown).

The next step of the study involved genotyping the ASD subjects and their siblings to investigate whether genetic factors such as one-carbon cycle polymorphisms could be related to DNA methylation. Not having observed any statistically significant difference between the ASD group and the SIBLINGS group, the analysis of *LINE-1* methylation levels stratified by genotype could show a correlation between genetic and epigenetic factors in contributing to susceptibility to ASD.

In this analysis we considered 7 genes with 8 polymorphisms, namely: *MTHFR* C677T rs1801133, *MTHFR* A1298C rs1801131, *DHFR* 19bp rs70991108, *CBS* 68bp rs876657421, *DNMT1* rs2228612, *DNMT3A* rs734693, *DNMT3B* rs1569686, and *DNMT3L* rs2070565.

Figures 27–34 and tables 20–27 show *LINE-1* sequence methylation results stratified by genotype in the ASD and SIBLINGS groups. Furthermore, we performed an intra-case analysis of *LINE-1* methylation levels, stratified by genotype. Genetic analyses that did not show statistically significant results or at least those tending towards significance are not shown.

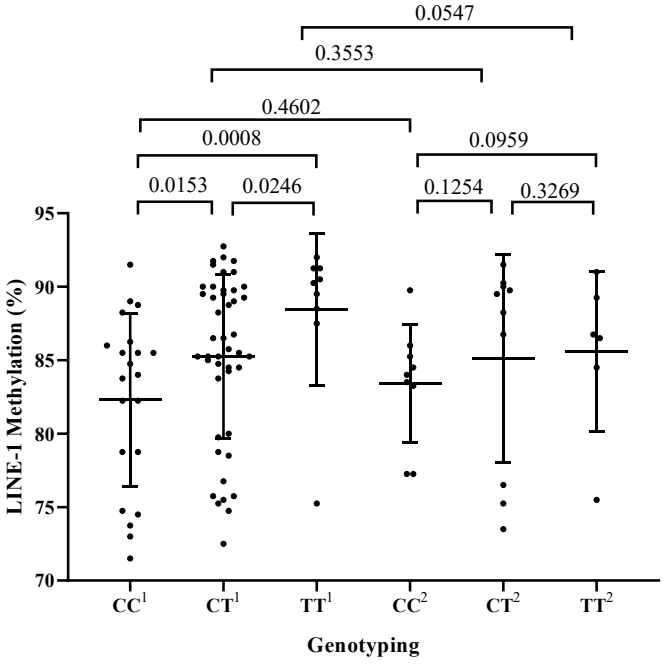


Figure 27. *LINE-1* methylation stratified by *MTHFR* C677T gene (rs1801133). The dot plots display *LINE-1* methylation expressed in %. Each graph shows the mean and SD, while the p-value between the analysed categories is reported at the top. At the superscript, 1 indicates the ASD group while 2 indicates the SIBLINGS group.

Table 20. *LINE-1* methylation stratified by *MTHFR* C677T gene (rs1801133)

	***MTHFR* C677T (rs1801133)**	**CC**	**CT**	**TT**
ASD (n=75)	**Subject n (%)**	21 (28)	45 (60)	9 (12)
	***LINE-1* Methylation (%)**			
	Minimum	71.50	72.50	75.25
	25% Percentile	76.75	81.88	88.00
	Median	**84.00**	**85.75**	**90.25**
	75% Percentile	86.13	89.75	91.25
	Maximum	91.50	92.75	92.00
	Mean±SD	**82.30±5.90**	**85.26±5.60**	**88.44±5.15**
CC *vs* CT	***p*-value**	**0.0153**		
CT *vs* TT		**0.0246**		
CC *vs* TT		**0.0008**		
SIBLINGS (n=25)	**Subject n (%)**	9 (36)	10 (40)	6 (24)
	***LINE-1* Methylation (%)**			
	Minimum	77.25	73.50	75.50
	25% Percentile	80.25	76.19	82.25
	Median	**84.00**	**88.88**	**86.63**
	75% Percentile	85.63	90.06	89.69
	Maximum	89.75	91.50	91.00
	Mean±SD	**83.42±4.00**	**85.13±7.08**	**85.58±5.44**
CC *vs* CT	***p*-value**	0.125		
CT *vs* TT		0.327		
CC *vs* TT		0.096		
ASD *vs* SIBLINGS	***p*-value**	0.460	0.355	**0.0547**

Statistically significant p-values ($p < 0.05$) are indicated in bold.

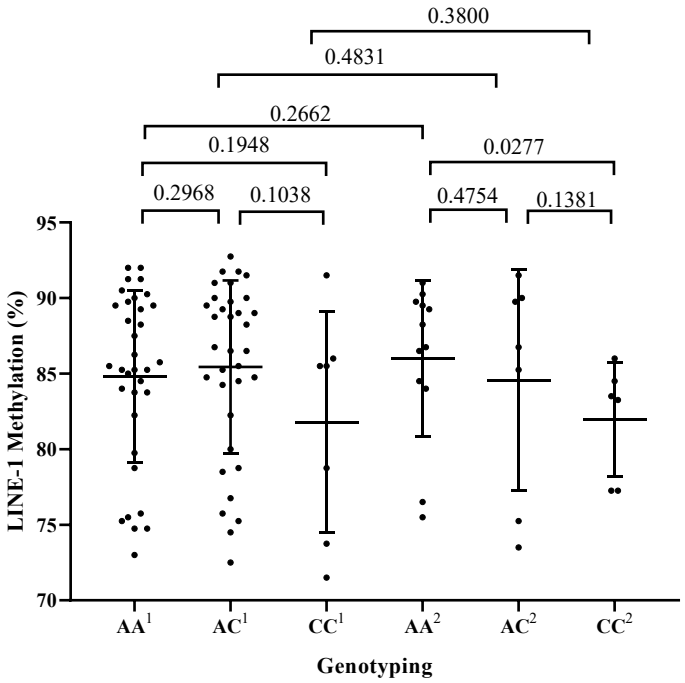


Figure 28. *LINE-1* methylation stratified by *MTHFR* A1298C gene (rs1801131). The dot plots display *LINE-1* methylation expressed in %. Each graph shows the mean and SD, while the p-value between the analysed categories is reported at the top. At the superscript, 1 indicates the ASD group while 2 indicates the SIBLINGS group.

Table 21. *LINE-1* methylation stratified by *MTHFR* A1298C gene (rs1801131)

	***MTHFR* A1298C (rs1801131)**	**AA**	**AC**	**CC**
ASD (n=75)	**Subject n (%)**	34 (45)	34 (45)	7 (10)
	***LINE-1* Methylation (%)**			
	Minimum	73.00	72.50	71.50
	25% Percentile	81.63	81.69	73.75
	Median	**85.38**	**86.63**	**85.50**
	75% Percentile	89.56	89.81	86.00
	Maximum	92.00	92.75	91.50
	Mean±SD	**84.81±5.70**	**85.44±5.72**	**81.79±7.29**
AA *vs* AC		0.297		
AC *vs* CC	***p*-value**	0.104		
AA *vs* CC		0.195		
SIBLINGS (n=25)	**Subject n (%)**	12 (16)	7 (28)	6 (24)
	***LINE-1* Methylation (%)**			
	Minimum	75.50	73.50	77.25
	25% Percentile	84.13	75.25	77.25
	Median	**87.50**	**86.75**	**83.38**
	75% Percentile	89.69	90.00	84.88
	Maximum	91.00	91.50	86.00
	Mean±SD	**85.98±5.16**	**84.57±7.29**	**81.96±3.77**
AA *vs* AC		0.475		
AC *vs* CC	***p*-value**	0.138		
AA *vs* CC		**0.028**		
ASD *vs* SIBLINGS	***p*-value**	0.266	0.483	0.380

Statistically significant p-values ($p < 0.05$) are indicated in bold.

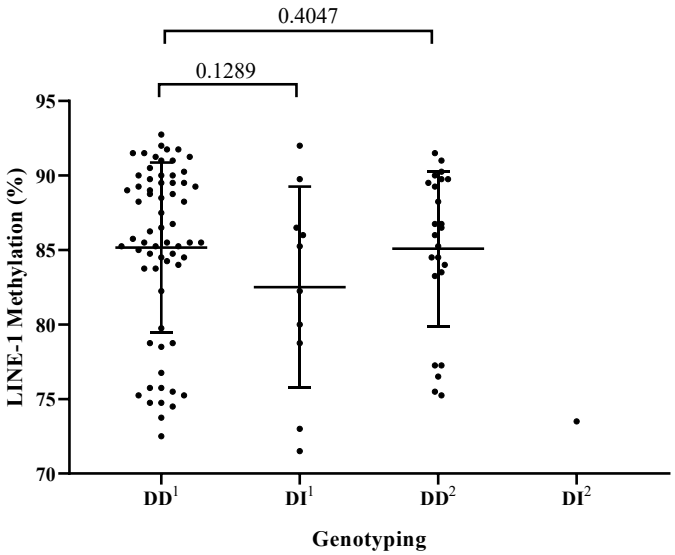


Figure 30. *LINE-1* methylation stratified by *CBS* 68 bp gene (rs876657421). The dot plots display *LINE-1* methylation expressed in %. Each graph shows the mean and SD, while the p-value between the analysed categories is reported at the top. At the superscript, 1 indicates the ASD group while 2 indicates the SIBLINGS group.

Table 23. *LINE-1* methylation stratified by *CBS* 68 bp gene (rs876657421)

	***CBS* 68 bp (rs876657421)**	**DD**	**DI**
ASD (n=75)	**Subject n (%)**	65 (87)	10 (13)
	***LINE-1* Methylation (%)**		
	Minimum	72.50	71.50
	25% Percentile	83.00	77.31
	Median	**85.75**	**83.75**
	75% Percentile	89.63	87.31
	Maximum	92.75	92.00
	Mean±SD	**85.17±5.70**	**82.50±6.75**
DD *vs* DI	***p*-value**	0.129	
SIBLINGS (n=25)	**Subject n (%)**	24 (96)	1 (4)
	***LINE-1* Methylation (%)**		
	Minimum	75.25	
	25% Percentile	83.31	
	Median	**86.25**	**73.50**
	75% Percentile	89.69	
	Maximum	91.50	
	Mean±SD	**85.08±5.19**	**73.50**
DD *vs* DI	***p*-value**	-	
ASD *vs* SIBLINGS	***p*-value**	0.405	

Statistically significant p-values ($p < 0.05$) are indicated in bold.

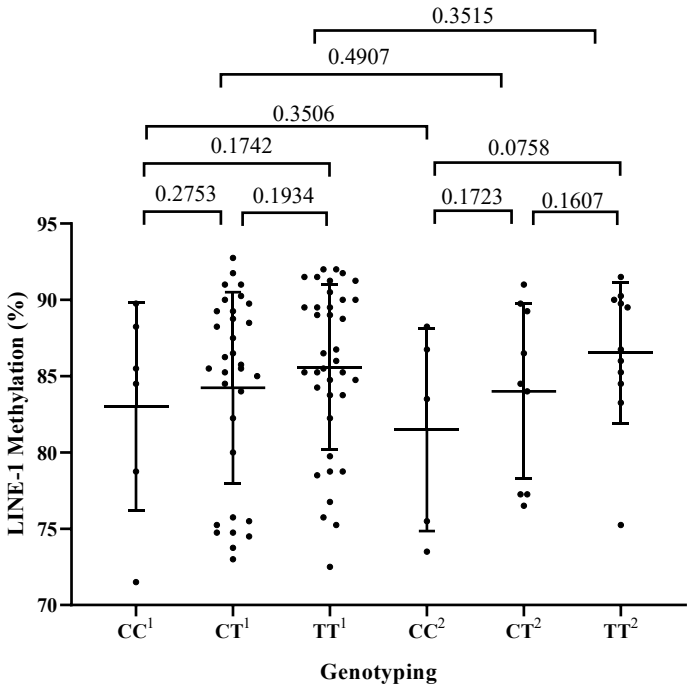


Figure 32. *LINE-1* methylation stratified by *DNMT3A* gene (rs734693). The dot plots display *LINE-1* methylation expressed in %. Each graph shows the mean and SD, while the p-value between the analysed categories is reported at the top. At the superscript, 1 indicates the ASD group while 2 indicates the SIBLINGS group.

Table 25. *LINE-1* methylation stratified by *DNMT3A* gene (rs734693)

	***DNMT3A* (rs734693)**	**CC**	**CT**	**TT**
ASD (n=75)	**Subject n (%)**	6 (8)	32 (43)	37 (49)
	***LINE-1* Methylation (%)**			
	Minimum	71.5	73	72.5
	25% Percentile	76.94	76.81	83
	Median	**85**	**85.63**	**86**
	75% Percentile	88.63	89.25	90
	Maximum	89.75	92.75	92
	Mean±SD	**83.04±6.81**	**84.24±6.26**	**85.59±5.40**
CC *vs* CT		0.275		
CT *vs* TT	***p*-value**	0.193		
CC *vs* TT		0.174		
SIBLINGS (n=25)	**Subject n (%)**	5 (20)	9 (36)	11 (44)
	***LINE-1* Methylation (%)**			
	Minimum	73.5	76.5	75.25
	25% Percentile	74.5	77.25	84.5
	Median	**83.5**	**84.5**	**86.75**
	75% Percentile	87.5	89.5	90
	Maximum	88.25	91	91.5
	Mean±SD	**81.50±6.65**	**84.00±5.74**	**86.55±4.62**
CC *vs* CT		0.172		
CT *vs* TT	***p*-value**	0.160		
CC *vs* TT		0.0758		

ASD *vs* SIBLINGS	***p*-value**	0.351	0.491	0.351

Statistically significant p-values ($p < 0.05$) are indicated in bold.

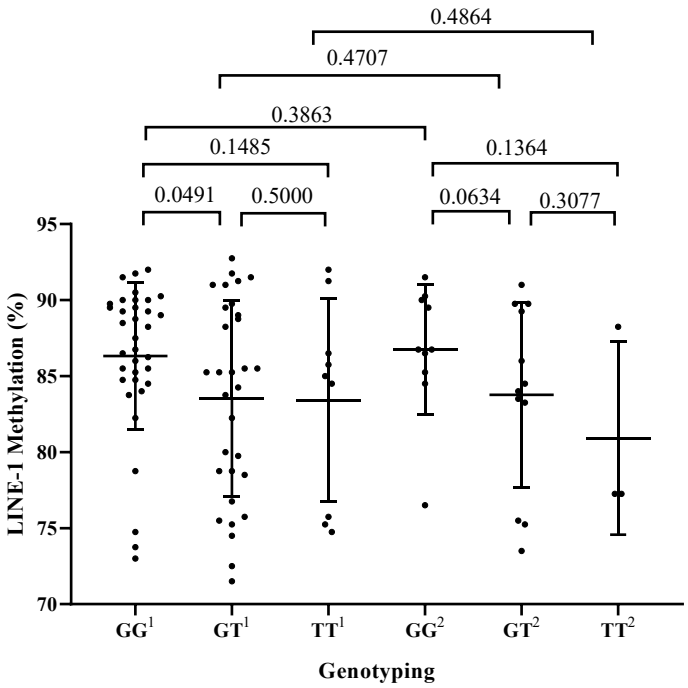


Figure 33. *LINE-1* methylation stratified by *DNMT3B* gene (rs1569686). The dot plots display *LINE-1* methylation expressed in %. Each graph shows the mean and SD, while the p-value between the analysed categories is reported at the top. At the superscript, 1 indicates the ASD group while 2 indicates the SIBLINGS group.

Table 26. *LINE-1* methylation stratified by *DNMT3B* gene (rs1569686)

	***DNMT3B* (rs1569686)**	**GG**	**GT**	**TT**
ASD (n=75)	**Subject n (%)**	35 (47)	31 (41)	9 (12)
	***LINE-1* Methylation (%)**			
	Minimum	73	71.5	74.75
	25% Percentile	84.75	78.5	75.5
	Median	**87.5**	**85.25**	**85**
	75% Percentile	89.75	89.5	88.88
	Maximum	92	92.75	92
	Mean±SD	**86.32±4.81**	**83.52±6.44**	**83.42±6.65**
GG *vs* GT	***p*-value**	**0.049**		
GT *vs* TT		0.500		
GG *vs* TT		0.148		
SIBLINGS (n=25)	**Subject n (%)**	10 (40)	12 (48)	3 (12)
	***LINE-1* Methylation (%)**			
	Minimum	76.5	73.5	77.25
	25% Percentile	85.06	77.44	77.25
	Median	**86.75**	**84.25**	**77.25**
	75% Percentile	90.06	89.63	88.25
	Maximum	91.5	91	88.25

	Mean±SD	**86.75±4.29**	**83.77±6.08**	**80.92±6.35**
GG *vs* GT		0.0634		
GT *vs* TT	***p*-value**	0.308		
GG *vs* TT		0.136		
ASD *vs* SIBLINGS	***p*-value**	0.386	0.471	0.486

Statistically significant p-values ($p < 0.05$) are indicated in bold.

By evaluating the *LINE-1* methylation levels in relation to the polymorphisms considered, we were able to see that only in the case of the TT genotype (rare genotype) of the *MTHFR* C677T gene, individuals in the ASD group had statistically increased methylation levels compared to the SIBLINGS group (88.44% and 85.58%, respectively; $p = 0.054$. Figure 27 and table 20). In parallel, analysis of the methylation levels of the ASD group according to genotype showed that an increase in the T allele of the *MTHFR* C677T gene was associated with higher *LINE-1* methylation levels (figure 27 and table 20). Specifically, the CC genotype, the CT genotype, and the TT genotype had methylation percentages of 82.30%, 85.26%, and 88.44%, respectively (CC *vs* CT $p = 0.015$; CT *vs* TT $p = 0.025$; CC *vs* TT $p = 0.0008$). The *DNMT3B* gene polymorphism also showed a statistically significant difference (figure 33 and table 26). From figure 34 it is in fact easy to see how methylation levels are statistically increased in ASD patients with the GG genotype (86.32%) compared to the heterozygous condition (83.52%, $p = 0.049$). It is unfortunately more difficult to evaluate the condition of the TT genotype due to the small number of subjects presenting this genotype.

To delve deeper into the relationship between epigenetic factors and the genetic component of ASD patients, we performed an analysis using genetic models. Specifically, we stratified the ASD population for the different polymorphisms using the dominant and recessive model. In the first case the common homozygote is isolated and compared with the recessive homozygote added to the contribution of the heterozygote, whereas in the recessive model the recessive homozygote is isolated. This analysis allows us to highlight any genetic and epigenetic differences due to the contribution of the heterozygote. Table 28 shows the genetic analyses obtained using dominant and recessive models for 6 of the 8 polymorphisms examined in the present study. The *CBS* rs876657421 polymorphism and the *DNMT1* rs2228612 polymorphism were excluded from the analysis because no rare homozygous individuals, ins/ins-genotype and CC genotype, respectively, were found in the ASD group.

Table 28. *LINE-1* Methylation in ASD group stratified by Genetic Model

***MTHFR* C677T rs1801133**			
Genetic model	**Subject n (%)**	**mean±SD**	**p-value**
CC *vs* TT+CT	21 (28) *vs* 54 (72)	82.3±5.9 *vs* 85.8±5.6	**0.0039**
TT *vs* CC+CT	9 (12) *vs* 66 (88)	88.4±5.1 *vs* 84.3±5.8	**0.0061**
***MTHFR* A1298C rs1801131**			
Genetic model	**Subject n (%)**	**mean±SD**	**p-value**
AA *vs* CC+AC	34 (45) *vs* 41 (55)	84.8±5.7 *vs* 84.8±6.1	0.423
CC *vs* AA+AC	7 (10) *vs* 68 (90)	81.8±7.3 *vs* 85.1±5.7	0.131
***DHFR* rs70991108**			
Genetic model	**Subject n (%)**	**mean±SD**	**p-value**
II *vs* DD+ID	25 (33) *vs* 50 (67)	84.5±5.4 *vs* 84.9±5.6	0.291
DD *vs* II+ID	11 (15) *vs* 64 (85)	84.5±7.7 *vs* 84.9±5.6	0.43985
***DNMT3A* rs734693**			
Genetic model	**Subject n (%)**	**mean±SD**	**p-value**
CC *vs* TT+CT	37 (49) *vs* 38 (51)	83.0±6.8 *vs* 85.0±5.8	0.206
TT *vs* CC+CT	6 (8) *vs* 69 (92)	85.6±5.4 *vs* 84.1±6.3	0.146
***DNMT3B* rs1569686**			
Genetic model	**Subject n (%)**	**mean±SD**	**p-value**
GG *vs* TT+GT	35 (47) *vs* 40 (53)	86.3±4.8 *vs* 83.5±6.4	**0.040**
TT *vs* GG+GT	9 (12) *vs* 66 (88)	83.4±6.6 *vs* 85.0±5.8	0.280
***DNMT3L* rs2070565**			
Genetic model	**Subject n (%)**	**mean±SD**	**p-value**
TT *vs* CC+TC	10 (13) *vs* 65 (87)	85.9±3.2 *vs* 84.6±6.2	0.422
CC *vs* TT+TC	26 (35) *vs* 49 (65)	84.5±6.3 *vs* 85.0±5.7	0.334

Statistically significant p-values ($p < 0.05$) are indicated in bold.

LINE-1 methylation analysis of the ASD group stratified by genetic model showed statistically significant differences for both *MTHFR* C677T rs1801133 and *DNMT3B* rs1569686 (table 28). Specifically, the analysis of *MTHFR* C677T rs1801133 showed a statistically significant difference for both genetic models (CC *vs* TT+CT, $p = 0.0039$ and TT *vs* CC+CT, $p = 0.0061$. Table 28) because the levels of *LINE-1* methylation observed in single genotype combinations increased with increasing T allele (figure 27 and table 20). In contrast, genetic analysis of *DNMT3B* rs1569686 highlighted a statistically significant difference in *LINE-1* methylation levels for the dominant model (GG *vs* TT+GT, $p = 0.040$. Table 28). This result shows that the TT homozygote contributes to increasing the epigenetic difference observed by the comparison between ASD individuals with GG e and GT genotype (figure 23 and table 26).

Intra-Case Analysis of *LINE-1* Methylation stratified by Clinical Data

To further explore the impact of epigenetic changes on ASD, an intra-case analysis was conducted by stratifying ASD patients with both key clinical data and comorbidities. The aim is to investigate if ASD subgroups with more severe phenotype showed differences in the degree of *LINE-1* methylation, indicative of a different degree of genomic stability.

Figure 35 and table 29 show *LINE-1* methylation levels stratified by severity for the six key clinical data considered in the analysis.

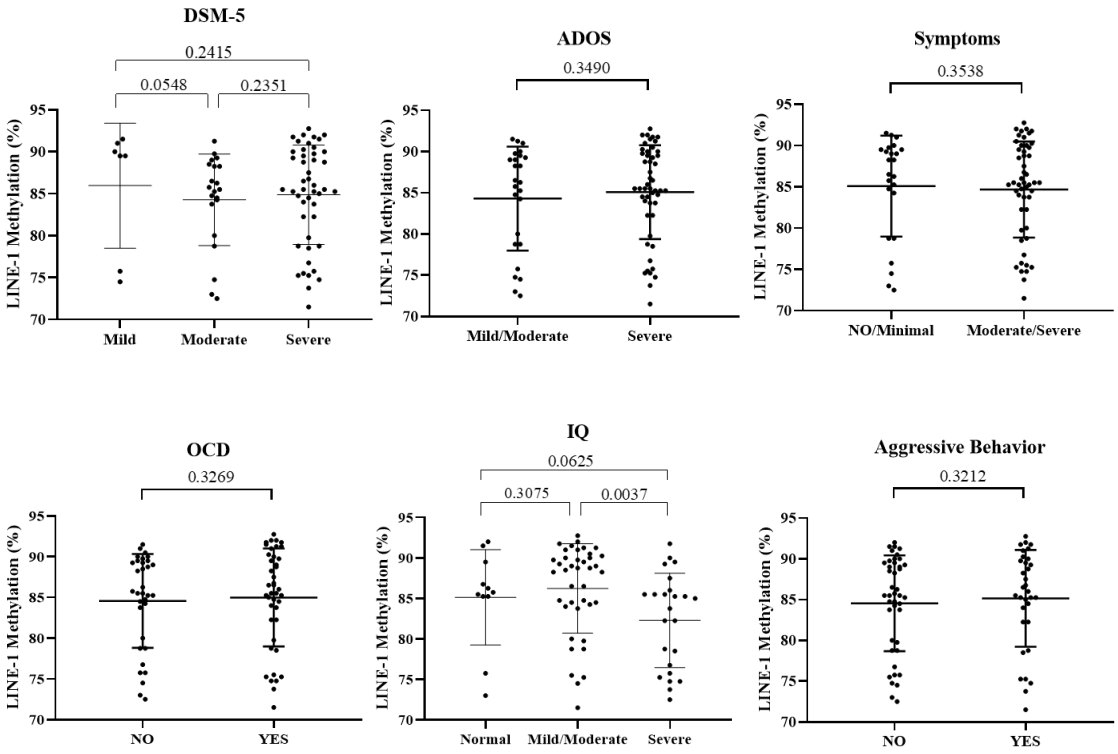


Figure 35. *LINE-1* methylation stratified by Principal Clinical Data. The dot plots display *LINE-1* methylation expressed in %. Each graph shows the mean and SD, while the p-value between the analysed categories is reported at the top.

Table 29. *LINE-1* methylation stratified by Principal Clinical Data

DSM-5	MILD	MODERATE	SEVERE
Subject n (%)	7 (9)	21 (28)	47 (63)
***LINE-1* Methylation (%)**			
Minimum	74.50	72.50	71.50
25% Percentile	75.80	81.90	79.80
Median	**89.50**	**85.50**	**85.50**
75% Percentile	91.00	88.40	90.00
Maximum	91.50	91.30	92.80
Mean±SD	**85.96±7.45**	**84.27±5.45**	**84.88±5.91**
***p*-value**			

MILD *vs* MODERATE	**0.055**		
MODERATE *vs* SEVERE	0.235		
MILD *vs* SEVERE	0.242		
ADOS	**MILD/MODERATE**	**SEVERE**	
Subject n (%)	25 (33)	50 (67)	
***LINE-1* Methylation (%)**			
Minimum	72.50	71.50	
25% Percentile	78.75	82.25	
Median	**86.25**	**85.50**	
75% Percentile	89.38	89.81	
Maximum	91.50	92.75	
Mean±SD	**84.30±3.30**	**85.07±5.69**	
***p*-value**			
MILD/MODERATE *vs* SEVERE	0.349		
SYMPTOMS	**NO/MINIMAL**	**MODERATE/SEVERE**	
Subject n (%)	24 (32)	51 (68)	
***LINE-1* Methylation (%)**			
Minimum	72.50	71.50	
25% Percentile	80.13	80.00	
Median	**87.38**	**85.50**	
75% Percentile	89.50	89.75	
Maximum	91.50	92.75	
Mean±SD	**85.09±6.1**	**84.68±5.81**	
***p*-value**			
NO/MINIMAL *vs* MODERATE/SEVERE	0.354		
OCD	**NO**	**YES**	
Subject n (%)	33 (44)	42 (56)	
***LINE-1* Methylation (%)**			
Minimum	72.50	71.50	
25% Percentile	79.38	81.63	
Median	**85.50**	**85.75**	
75% Percentile	89.38	90.06	
Maximum	91.50	92.75	
Mean±SD	**84.58±5.76**	**84.99±6.01**	
***p*-value**			
NO *vs* YES	0.327		
IQ	**NORMAL**	**MILD/MODERATE**	**SEVERE**
Subject n (%)	11 (15)	40 (53)	24 (32)
***LINE-1* Methylation (%)**			
Minimum	73.00	71.50	72.50
25% Percentile	85.25	84.06	76.00
Median	**85.75**	**88.63**	**84.38**
75% Percentile	89.50	90.19	85.88
Maximum	92.00	92.75	91.75
Mean±SD	**85.14±5.89**	**86.24±5.52**	**82.29±5.83**
***p*-value**			
NORMAL *vs* MILD/MODERATE	0.3075		
MILD/MILDMODERATE *vs* SEVERE	**0.0037**		
NORMAL *vs* SEVERE	0.0625		
Aggressive Behaviour	**NO**	**YES**	
Subject n (%)	42 (56)	33 (44)	
***LINE-1* Methylation (%)**			
Minimum	72.5	71.5	
25% Percentile	79.5	82.25	
Median	**85.5**	**86.0**	
75% Percentile	89.5	89.88	
Maximum	92.0	92.75	

Mean±SD	**84.55±5.87**	**85.15±5.93**
p-value		
NO *vs* YES	0.321	

Statistically significant p-values ($p < 0.05$) are indicated in bold.

Stratification based on DSM-5 revealed that patients with a mild phenotype had higher methylation levels compared to the moderate phenotype (85.96% and 84.27%, respectively; $p = 0.055$), although this assessment is further to be confirm due to the limited number of patients with a mild phenotype. An interesting result was obtained by stratifying the ASD group by intelligence quotient to investigate whether the increase in intellectual disability was associated with a different percentage of global DNA methylation. Indeed, patients with a severe phenotype showed reduced *LINE-1* methylation levels compared to the mild/moderate phenotype ($p = 0.0037$) and the normal phenotype ($p = 0.0625$). Analysis of *LINE-1* methylation levels of the ASD group after stratification for ADOS, symptoms, OCD and aggression did not yield statistically significant results ($p = 0.35$, $p = 0.35$ and $p = 0.33$, respectively).

We also conducted epigenetic investigation considering the comorbidities recognized in patients with ASD. We then analysed the *LINE-1* methylation levels of the ASD group stratified by these accessory disorders that aggravate the disease course. The results of the analysis are shown in figure 36 and table 30.

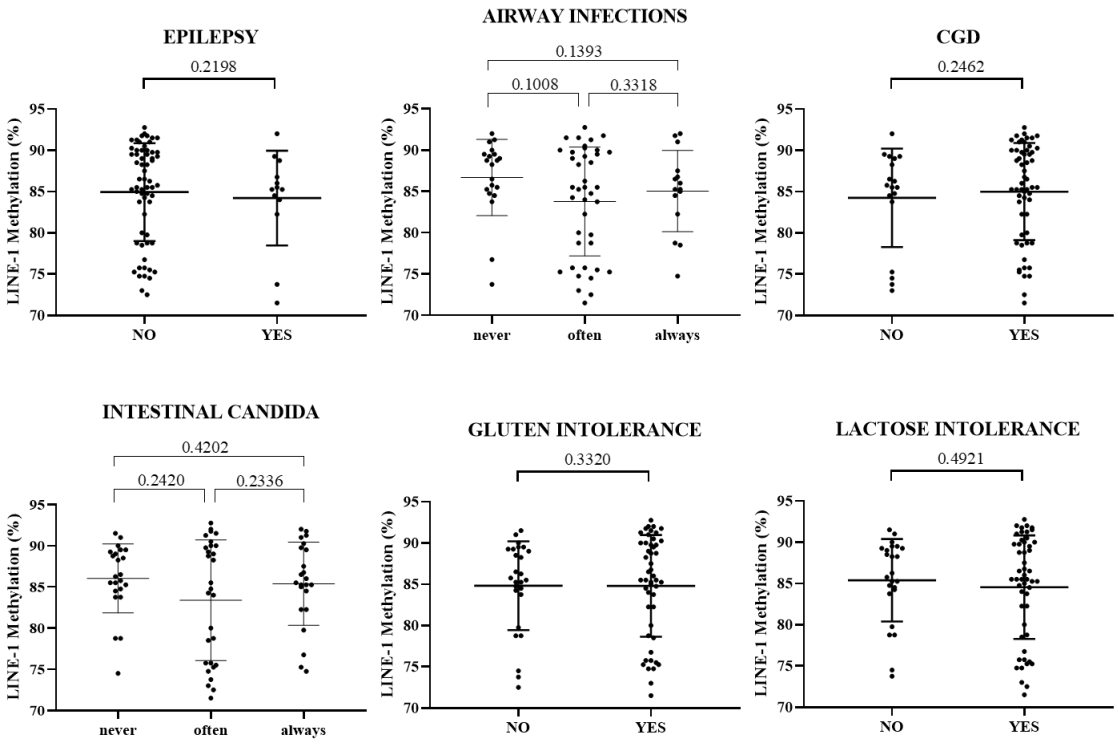


Figure 36. *LINE-1* methylation stratified by Comorbidities. The dot plots display *LINE-1* methylation expressed in %. Each graph shows the mean and SD, while the p-value between the analysed categories is reported at the top.

Table 30. *LINE-1* methylation stratified by Comorbidities

EPILEPSY	**NO**	**YES**	
Subject n (%)	62 (83)	13 (17)	
***LINE-1* Methylation (%)**			
Minimum	72.5	71.5	
25% Percentile	79.5	83.13	
Median	**86**	**85.25**	
75% Percentile	89.81	87.75	
Maximum	92.75	92	
Mean±SD	**84.94±5.93**	**84.21±5.74**	
***p*-value**			
NO *vs* *YES*	0.22		
AIRWAY INFECTIONS	**NEVER**	**OFTEN**	**ALWAYS**
Subject n (%)	20 (27)	40 (53)	15 (20)
***LINE-1* Methylation (%)**			
Minimum	73.75	71.5	74.75
25% Percentile	84.88	76.5	82.25
Median	**88.5**	**85.38**	**85.25**
75% Percentile	89.5	89.75	87.5
Maximum	92	92.75	92
Mean±SD	**86.69±4.62**	**83.79±6.59**	**85.05±4.91**
***p*-value**			
NEVER *vs* OFTEN	0.101		
OFTEN *vs* ALWAYS	0.332		
NEVER *vs* ALWAYS	0.139		
CHRONIC GASTROINTESTINAL DISORDERS	**NO**	**YES**	
Subject n (%)	18 (24)	57 (76)	
***LINE-1* Methylation (%)**			
Minimum	73	71.5	
25% Percentile	81.63	79.88	
Median	**85.63**	**85.5**	
75% Percentile	89.06	90	
Maximum	92	92.75	
Mean±SD	**84.24±5.96**	**85.0±5.88**	
***p*-value**			
NO *vs* *YES*	0.246		
INTESTINAL CANDIDA	**NEVER**	**OFTEN**	**ALWAYS**
Subject n (%)	23 (30.6)	29 (38.7)	23 (30.6)
***LINE-1* Methylation (%)**			
Minimum	74.5	71.5	74.75
25% Percentile	84.5	75.63	82.25
Median	**86.25**	**84.75**	**85.5**
75% Percentile	89.25	90	89.75
Maximum	91.5	92.75	92
Mean±SD	**86.03±4.19**	**83.39±7.32**	**85.39±5.05**
***p*-value**			
NEVER *vs* OFTEN	0.242		
OFTEN *vs* ALWAYS	0.234		
NEVER *vs* ALWAYS	0.420		

GLUTEN INTOLERANCE	NO	YES
Subject n (%)	26 (35)	49 (65)
***LINE-1* Methylation (%)**		
Minimum	72.5	71.5
25% Percentile	82.75	79.38
Median	**85.63**	**85.5**
75% Percentile	89.25	90
Maximum	91.5	92.75
Mean±SD	**84.83±5.38**	**84.81±6.17**
***p*-value**		
NO *vs YES*	0.332	
LACTOSE INTOLERANCE	**NO**	**YES**
Subject n (%)	24 (32)	51 (68)
***LINE-1* Methylation (%)**		
Minimum	73.75	71.5
25% Percentile	83.88	78.75
Median	**86**	**85.5**
75% Percentile	89.25	90
Maximum	91.5	92.75
Mean±SD	**85.39±4.99**	**84.54±6.27**
***p*-value**		
NO *vs YES*	0.492	

Analysis of the ASD group stratified by comorbidities showed no statistically significant results (all p-values obtained were greater than 0.05).

Correlation of *LINE-1* methylation between parent and child

To further evaluate whether the degree of global DNA methylation is implicated in the predisposition to develop autism, we conducted a correlation analysis of *LINE-1* methylation between parents and children, with the aim of identifying possible correlations and differences between parents and children with ASD and siblings. The correlation was performed by initially comparing the DNA methylation level of all children with that of both mothers and fathers. Afterwards, the same approach was also applied to the comparison of parents with both patients and unaffected children.

In the first analysis, we assessed parent-child correlation to explore *LINE-1* methylation in the entire cohort of children (Figure 37).

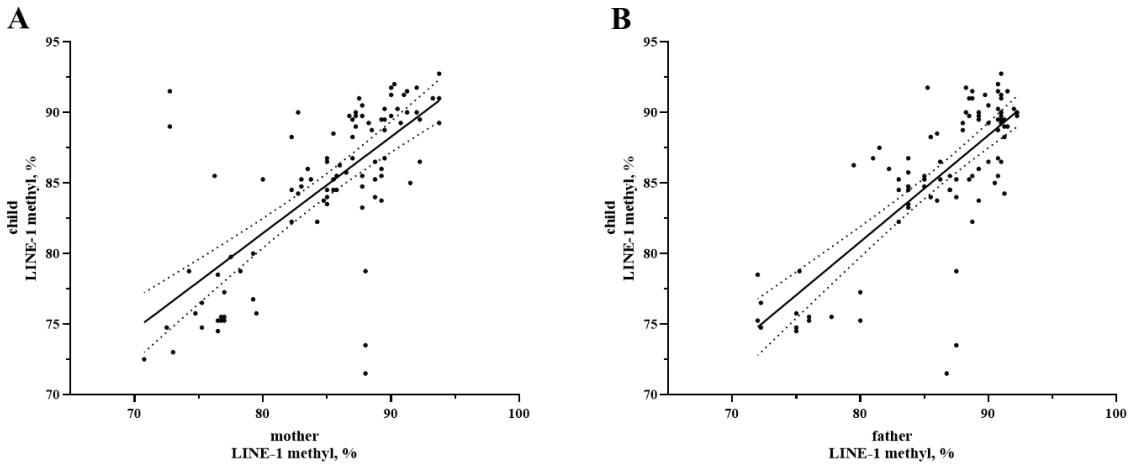


Figure 37. Correlation of *LINE-1* methylation between parents and children. The scatter plots display *LINE-1* methylation expressed in %. Each graph shows the regression line and confidence intervals. **(A)**: correlation analysis between mothers and children, while **(B)**: correlation analysis between fathers and children.

Table 31. Correlation graphs of *LINE-1* methylation between parents and children

	Mother *vs* Child	**Father *vs* Child**
Number of XY Pairs	95	90
Regression Equation		
Slope (95% CI)	0.68 (0.54-0.82)	0.75 (0.62-0.89)
Y-Intercept (95% CI)	26.7 (14.9-38.5)	20.4 (8.91-31.9)
Pearson r		
r (95% CI)	**0.7115** (0.59-0.80)	**0.7682** (0.66-0.84)
R^2	0.5063	0.5901
p-value	**<0.0001**	**<0.0001**

Statistically significant p-values ($p < 0.05$) are indicated in bold. CI: Confidence Intervals.

The results show a strong correlation of *LINE-1* methylation levels between children and both parents (table 31), $p < 0.0001$. The next step analyses parent-child correlation by stratifying children based on the presence of ASD to verify if the childASD group and the childSIBLINGS group exhibit different correlations in global DNA methylation.

Figure 39 illustrates *LINE-1* methylation correlations between mothers and children with ASD (n = 70) (figure 38A) and mothers and unaffected children (n = 25) (figure 38B). In figure 38C, the regression lines obtained from the previous correlation analyses are compared to better understand the differences between ASD children and siblings. Table 32 also presents the results of the Pearson correlation analysis.

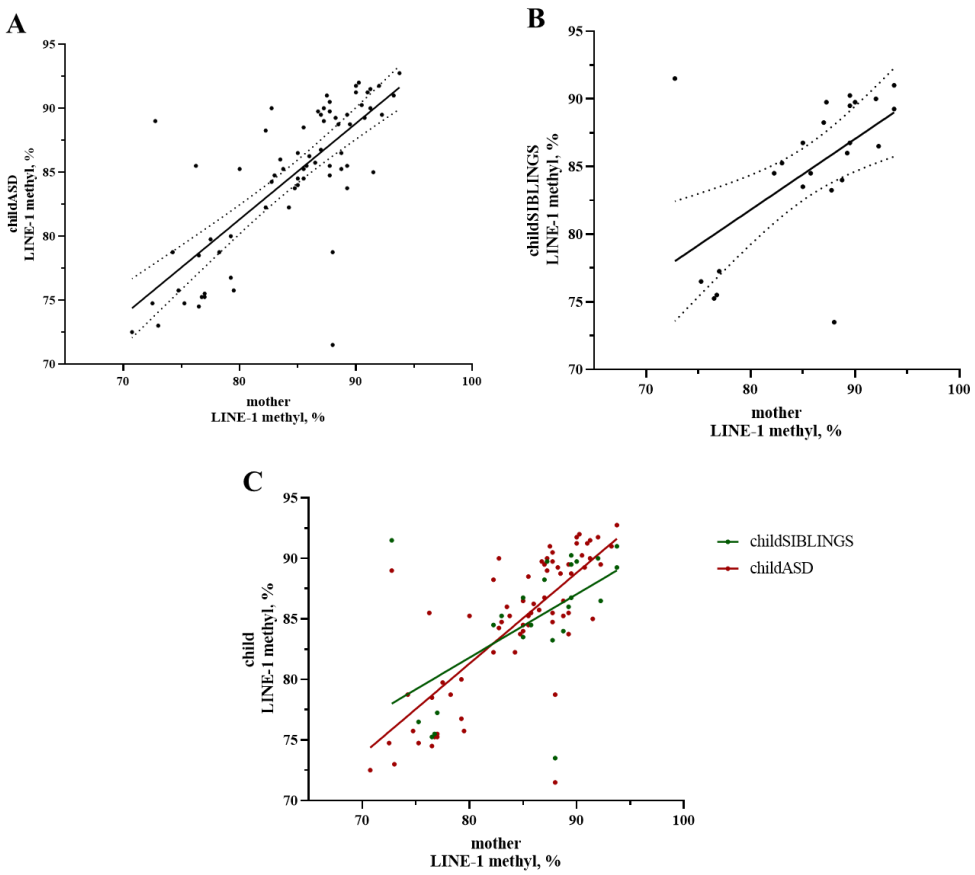


Figure 38. Correlation of *LINE-1* methylation between mother and child. The scatter plots display *LINE-1* methylation expressed in %. Each graph shows the regression line and confidence intervals. **(A)**: correlation analysis between mothers and children with ASD, **(B)**: correlation analysis between mother and siblings, **(C)**: comparison between A and B.

Table 32. Correlation of *LINE-1* methylation between mother and child

	Mother *vs* childASD	**Mother *vs* childSIBLINGS**
Number of XY Pairs	70	25
Regression Equation		
Slope (95% CI)	0.75 (0.60-0.90)	0.52 (0.21-0.84)
Y-Intercept (95% CI)	21.3 (8.15-34.4)	39.9 (12.8-67.0)
Pearson r		
r (95% CI)	**0.7609** (0.64-0.84)	**0.5813** (0.24-0.79)
R^2	0.5790	0.3379
p-value	**<0.0001**	**0.0023**
p intercepts	0.48	
p slope	0.15	

Statistically significant p-values ($p < 0.05$) are indicated in bold. CI: Confidence Intervals.

The analysis revealed a strong correlation in *LINE-1* methylation levels between mothers and ASD children (r = 0.76; p <0.0001). The correlation of global DNA methylation levels was also observed between mothers and unaffected children (r = 0.58; p = 0.0023), but the latter was lower than the former, confirming a greater correlation between mothers and autistic children. To confirm the distinct correlation between mothers and ASD children versus mothers and healthy children, we selected ASD patients (n = 21) who participated in the study together with their unaffected SIBLINGS (n = 25). The results are illustrated in figure 39 and table 33.

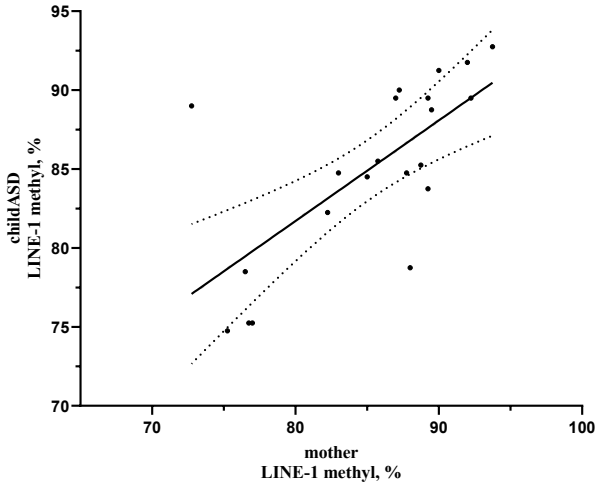


Figure 39. Correlation of *LINE-1* methylation between mother and ASD child. The scatter plots display *LINE-1* methylation expressed in %. Graph shows the regression line and confidence intervals.

Table 33. Correlation of *LINE-1* methylation between mother and ASD child

	Mother *vs* childASD
Number Of XY Pairs	21
Regression Equation	
Slope (95% CI)	0.64 (0.32-0.96)
Y-Intercept (95% CI)	30.7 (3.36-58.1)
Pearson r	
r (95% CI)	**0.6909** (0.37-0.86)
R2	0.4773
p-value	**0.0005**

Statistically significant p-values (p < 0.05) are indicated in bold. CI: Confidence Intervals.

This more rigorous analysis confirmed that the correlation in *LINE-1* methylation levels between mothers and ASD children (r = 0.6909; p = 0.0005; table 33) was higher than the correlation between mothers and unaffected SIBLINGS (r = 0.5813; p = 0.0023. Table 32).

After examining the mother-child correlation, we explored the correlation between *LINE-1* methylation levels in fathers and ASD children and between father and SIBLINGS. In this analysis, we used a smaller number of samples than in the mother-child correlation analysis, since the number of fathers (n = 64) was lower than the number of individuals with ASD. The correlation analyses between fathers and ASD children (n = 65) and between fathers and unaffected children (n = 25) are shown in figure 40 and table 34.

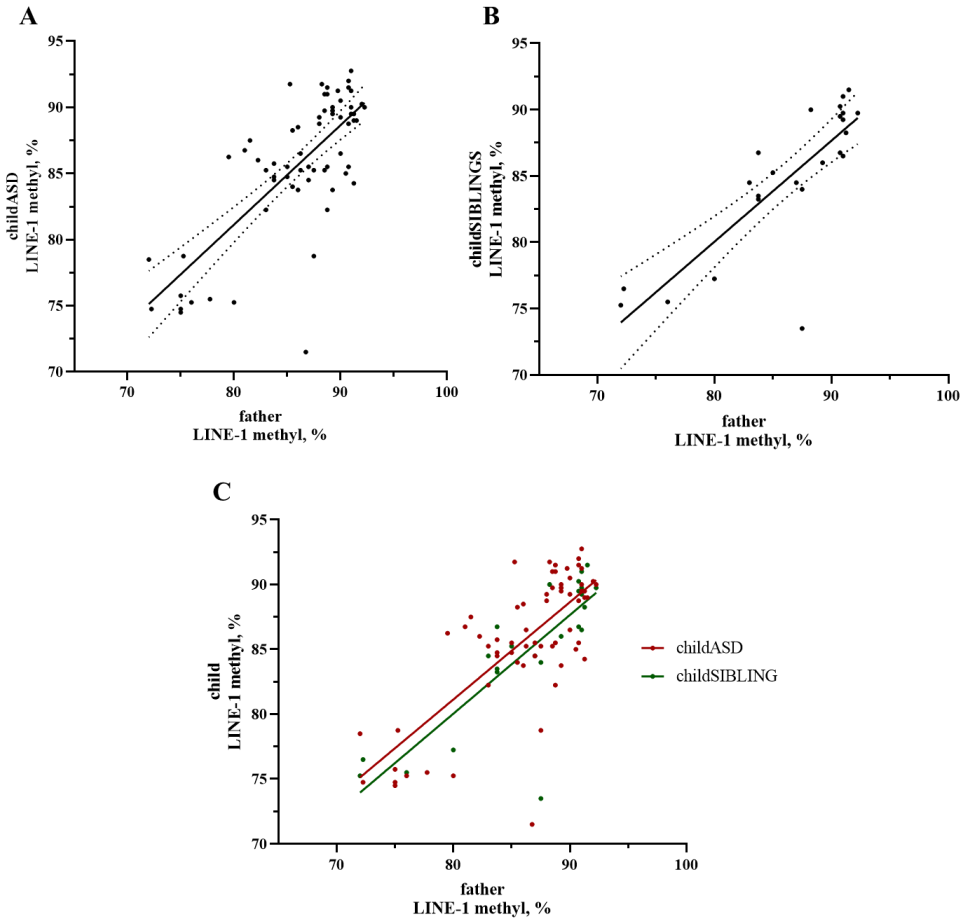


Figure 40. Correlation of *LINE-1* methylation between father and child. The scatter plots display *LINE-1* methylation expressed in %. Each graph shows the regression line and confidence intervals. **(A)**: correlation analysis between father and childASD, **(B)**: correlation analysis between father and childSIBLINGS, **(C)**: comparison between A and B.

Table 34. Correlation graphs of *LINE-1* methylation between father and child

	Father *vs* childASD	Father *vs* childSIBLINGS
Number of XY Pairs	65	25
Regression Equation		
Slope (95% CI)	0.75 (0.58-0.92)	0.76 (0.53-0.99)
Y-intercept (95% CI)	21.0 (6.59-35.4)	19.1 (-0.6-38.8)
Pearson r		
r (95% CI)	**0.75** (0.62-0.84)	**0.82** (0.63-0.92)
R^2	0.5612	0.6735
P value (two-tailed)	**<0.0001**	**<0.0001**
p intercepts	0.20	
p slope	0.94	

Statistically significant p-values ($p < 0.05$) are indicated in bold. CI: Confidence Intervals.

As shown in figure 40 and table 34, the correlation of *LINE-1* methylation levels is high both between fathers and unaffected children ($r = 0.8207$; $p < 0.0001$) and between fathers and ASD children ($r = 0.7491$; $p < 0.0001$).

The results confirm a strong correlation in *LINE-1* methylation levels between parents and ASD children, as well as between parents and the healthy siblings group.

We also analysed the parent-offspring correlation considering genetic transmission, but this analysis did not produce significant results (data not shown).

Principal Component Analysis between ASD and SIBLINGS groups

PCA was conducted to explore the extensive data obtained during the experimental phase of the study. PCA, also known as the Karhunen-Loève transform, is a data simplification technique used in multivariate statistics. The technique aims to reduce the number of variables that describe a set of data, minimizing the loss of information as much as possible. PCA specifically aims to maximize variance by calculating the weight to assign to each original variable to concentrate them into one or more new variables (called principal components), which constitute a linear combination of the original variables.

The database used for PCA included 75 ASD patients and 25 healthy individuals (SIBLINGS group). After normalizing the data, the introduced variables were as follows: age, sex, 11 cytokines (G-CSF, IL-1β, IL-1RA, IL-4, IL-5, IL-7, IL-8, IL-10, IL-12(p40), MCP-1, and TNF-α), *LINE-1* methylation, and 8 polymorphisms belonging to 7 different genes (*MTHFR* C677T, *MTHFR* A1298C, *DHFR* 19bp, *CBS* 68bp, *DNMT1*, *DNMT3A*, *DNMT3B*, and *DNMT3L*).

The adequacy of the sample was confirmed by the analysis result using the Kaiser-Meyer-Olkin index (0.614) and Bartlett's test of sphericity (< 0.001). Subsequently, the first 9 components of the 22 proposed were extracted based on components that had an eigenvalue equal to or greater than 1 and ensured a variance greater than 60-80 % of the total variability, thus limiting the loss of information from the inserted variables. As shown in table 35, the first 9 components cumulatively explained 70.384 % of the total variance and had eigenvalues ranging from 1.001 to 4.162.

Table 35. Total variance explained of PCA with ASD e SIBLINGS groups

	Eigenvalues		
Component	**Total**	**% variance**	**% cumulative**
1	**4.162**	18.916	18.916
2	**1.957**	8.894	27.810
3	**1.749**	7.951	35.761
4	**1.646**	7.482	43.244
5	**1.451**	6.597	49.841
6	**1.278**	5.808	55.649
7	**1.208**	5.492	61.141
8	**1.033**	4.696	65.836
9	**1.001**	4.548	**70.384**
10	0.895	4.070	74.454
11	0.837	3.803	78.258

12	0.755	3.430	81.688
13	0.666	3.025	84.713
14	0.613	2.786	87.499
15	0.575	2.611	90.111
16	0.520	2.363	92.474
17	0.375	1.705	94.179
18	0.331	1.502	95.682
19	0.321	1.461	97.143
20	0.270	1.229	98.372
21	0.226	1.027	99.399
22	0.132	0.601	100.000

Each component generated by PCA is made up of a set of considered variables to which the analysis assigns different weights. Table 36 shows the weight assigned to each variable present in the first 9 principal components, considering the variables with coefficient > +0.3 and < -0.3. In contrast, in figure 41 it is shown how the 22 variables are distributed in space according to the first three PCs.

Table 36. Component matrix of PCA with ASD e SIBLINGS groups

	COMPONENT								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
TNF-α	**0.788**	0.109	-0.196	-0.168	-0.041	0.079	0.061	-0.058	0.175
G-CSF	**0.693**	0.204	-0.223	-0.125	-0.145	0.114	0.124	-0.066	**-0.341**
IL-1RA	**0.692**	-0.124	**0.452**	0.032	-0.040	-0.013	-0.079	0.096	-0.092
IL-4	**0.668**	0.078	0.128	0.018	**0.429**	0.093	-0.075	-0.234	0.096
IL-12(p40)	**0.627**	0.113	**0.430**	0.190	-0.209	-0.125	-0.157	0.018	0.040
IL-10	**0.542**	0.258	-0.225	0.267	**-0.391**	0.082	0.004	0.007	**-0.324**
IL-7	**0.521**	0.122	-0.266	0.025	**0.538**	0.052	0.103	-0.062	0.098
IL-1β	**0.507**	-0.128	**0.685**	0.068	0.224	-0.100	-0.185	-0.117	-0.114
MCP-1	**0.503**	0.294	**-0.418**	-0.133	-0.111	-0.068	-0.041	0.012	**0.310**
IL-5	**0.433**	-0.083	-0.099	**-0.387**	-0.034	0.258	-0.013	0.285	0.444
IL-8	0.273	0.228	-0.060	-0.018	0.068	-0.168	-0.058	**0.672**	**-0.316**
***MTHFR* C677T**	0.249	**-0.573**	-0.282	0.251	**0.373**	-0.040	0.123	0.145	-0.188
DNMT3L	0.117	0.222	0.267	0.239	-0.260	0.017	**0.676**	0.231	0.117
LINE-1	0.115	**-0.657**	0.188	-0.256	0.071	-0.170	0.246	0.284	0.108
DNMT3A	-0.027	0.295	0.115	**0.459**	**0.356**	**0.347**	0.033	-0.019	0.117
***DHFR* 19bp**	-0.054	-0.097	0.143	-0.296	0.012	**0.666**	-0.270	0.286	-0.079
DNMT1	-0.077	-0.275	0.086	**0.475**	-0.282	**0.394**	-0.100	0.111	0.265

Sex	-0.121	0.297	-0.019	0.130	**0.317**	**-0.467**	-0.047	0.288	0.226
DNMT3B	-0.214	0.201	-0.194	**0.475**	0.124	0.039	**-0.453**	0.270	0.016
***MTHFR* A1298C**	-0.247	**0.539**	**0.363**	-0.414	-0.082	-0.150	-0.232	0.041	0.119
***CBS* 68bp**	-0.282	**0.409**	0.276	0.130	0.194	0.226	**0.456**	0.035	0.027
Age	**-0.324**	0.240	0.054	**-0.425**	**0.333**	0.269	0.129	0.047	-0.312

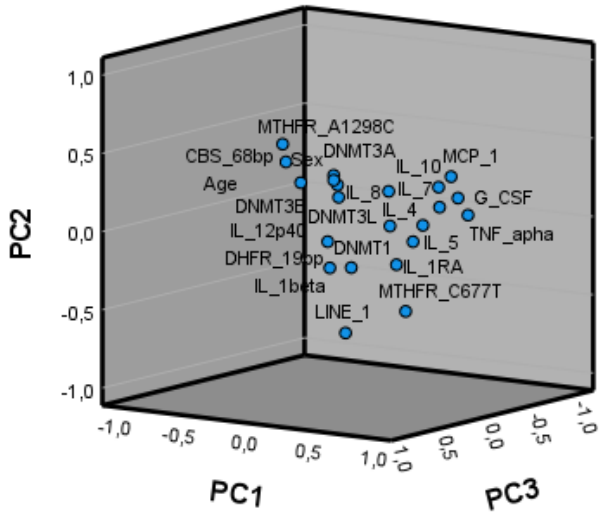


Figure 41. PCA result for the computed 22 variables of ASD and SIBLING groups: PC1, PC2 and PC3 loadings. Distribution in the rotated space of the 22 components considering the first three PCs. Plotted by SPSS (Statistics Version 22).

From table 36, it is possible to observe that in PC1 the cytokines, associated with each other, are predominant (highlighted in bold). Conversely, in PC2, other variables took on greater weight, revealing a new correlation. In particular, the correlation was present among *LINE-1* methylation, the *MTHFR* C677T rs1801133, *MTHFR* A1298C rs1801131 and *CBS* 68 bp rs876657421 genes (table 36). These two principal components were the most relevant in the analysis because they could explain a higher percentage of the total variance: 18.92 % and 8.89 % for PCA1 and PCA2, respectively (table 35). The results obtained from PCA were subjected to subsequent binary logistic regression analysis to identify which of the 9 principal components could best distinguish between individuals with ASD and the SIBLINGS groups.

Binary Logistic Regression between Cases and Controls: ASD *vs* CTR

Binary logistic regression is a probabilistic analysis that examines the different principal components of the study to identify variables that best explain the difference between cases and controls. It is a nonlinear regression model in which the dependent variable is dichotomous. This is a predictive analysis used in this case to explain the relationship between the probability of being affected or not (dependent binary variable) and the principal components of PCA (independent variables). Remembering that the principal components are made up of different weights of the various variables under study, the aim was to identify which variables could play a role in the diagnosis of ASD.

Specifically, for the binary logistic regression, the first 9 principal components of the PCA were examined, i.e., those components that explained 70.384% of the total cumulative variance. The outcome of the analysis was represented by the state of being affected or not. As shown in the table 37, the analysis highlighted 2 principal components out of the 9 analysed. In particular, PC6 was significant (p = 0.028; OR: 0.57 with CI: 0.35-0.94), and PC7 was borderline significant (p = 0.057; OR: 1.72 with CI: 0.98-3.04).

Table 37. Variables included in the binary logistic regression between ASD and SIBLINGS groups

PCA	B	SE	Wald	df	Sign.	OR	95% CI per OR	
							Inferior	Superior
1	-0.245	0.240	1.041	1	0.308	0.783	0.489	1.253
2	0.085	0.248	0.119	1	0.731	1.089	0.670	1.771
3	0.087	0.247	0.124	1	0.724	1.091	0.672	1.770
4	0.262	0.237	1.215	1	0.270	1.299	0.816	2.068
5	0.114	0.240	0.227	1	0.634	1.121	0.700	1.795
6	-0.557	0.254	4.818	1	**0.028**	0.573	0.349	0.942
7	0.547	0.287	3.629	1	**0.057**	1.729	0.984	3.036
8	0.183	0.277	0.437	1	0.508	1.201	0.698	2.067
9	0.195	0.250	0.610	1	0.435	1.215	0.745	1.983
Constant	1.283	0.270	22.515	1	0.000	3.608		

Statistically significant p-values ($p < 0.05$) are indicated in bold. B: Estimated regression coefficient; SE: Standard Error; Wald: statistic test; df: degree of freedom; Sign.: Significance; OR: Odd Ratio; CI: Confidence Intervals.

By comparing the significant components obtained from the binary logistic regression with the principal component matrix (table 36), we were able to verify that PC6 is characterized by associations between the variables *DNMT3A, DNMT1, DHFR* 19bp and sex, while PC7

was characterized by the strong association between *DNMT3L*, *DNMT3B*, and *CBS* 68bp (figure 42).

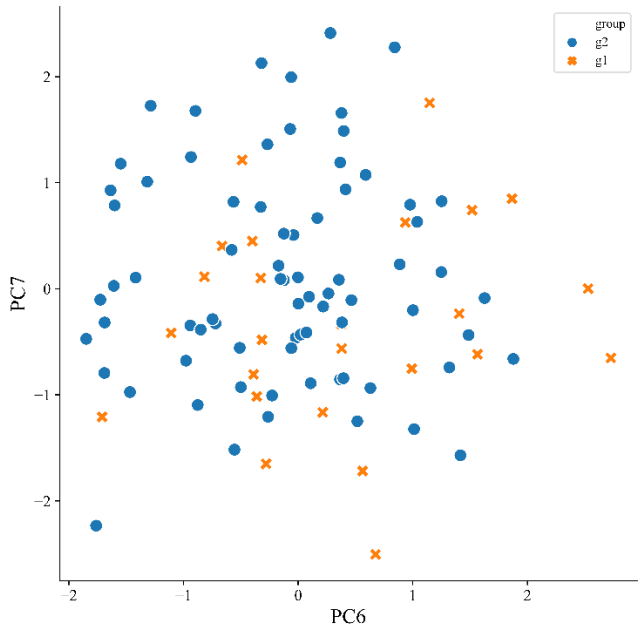


Figure 42. 2D-loading plot of the ASD and SIBLINGS scores (n = 100) based on PC6 and PC7. Blue dots represent ASD group (n=75), while orange crosses represent SIBLINGS group (n =25). Plotted by bioinformatics.com.cn/srplot.

Principal Component Analysis in ASD group

To explore variables associated with the ASD group, we conducted a PCA to identify which factors most represent the patient group. We performed a double PCA: the first analysis focused on biochemical, genetic, and epigenetic factors, while in the second PCA we added the patients' comorbidities to identify which variables had more weight in the analysis and were more strongly associated with the ASD group.

In the first PCA of the 75 ASD individuals, we considered age, sex, 11 cytokines (G-CSF, IL-1β, IL-1RA, IL-4, IL-5, IL-7, IL-8, IL-10, IL-12(p40), MCP-1, and TNF-α), *LINE-1* methylation, and 8 polymorphisms belonging to 7 different genes (*MTHFR* C677T, *MTHFR* A1298C, *DHFR* 19bp, *CBS* 68bp, *DNMT1*, *DNMT3A*, *DNMT3B*, and *DNMT3L*) as variables. The adequacy of the sample was confirmed through the Kaiser-Meyer-Olkin index ($p = 0.534$) and Bartlett's test of sphericity ($p < 0.001$). The analysis identified the top 10 components of the 22 proposed, as they had eigenvalues ranging from 1.006 to 3.477 and overall explained a total variance of 73.77% (table 38).

Table 38. Total variance explained by PCA with ASD group

	Eigenvalues		
Component	**Total**	**% variance**	**% cumulative**
1	**3.477**	15.804	15.804
2	**2.170**	9.864	25.668
3	**2.037**	9.260	34.928
4	**1.570**	7.138	42.066
5	**1.426**	6.482	48.549
6	**1.272**	5.783	54.332
7	**1.149**	5.223	59.554
8	**1.091**	4.960	64.515
9	**1.030**	4.682	69.197
10	**1.006**	4.574	**73.770**
11	0.840	3.819	77.589
12	0.825	3.750	81.340
13	0.701	3.186	84.525
14	0.630	2.866	87.391
15	0.586	2.663	90.054
16	0.490	2.226	92.280
17	0.409	1.857	94.137
18	0.379	1.725	95.862
19	0.336	1.528	97.389
20	0.259	1.176	98.565
21	0.221	1.003	99.567
22	0.095	0.433	100.000

The variables that make up the 10 PCs are shown in table 39, arbitrarily considering the predominant variables in each PC with a value greater than +0.3 and less than -0.3. In contrast, in figure 43 it is shown how the 22 variables are distributed in space according to the first three PCs.

Table 39. Component matrix of PCA with ASD group

	COMPONENT									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
IL-12(p40)	**0.675**	**0.449**	0.131	-0.07	-0.206	0.035	-0.173	-0.106	0.009	0.020
TNF-α	**0.667**	-0.280	0.032	-0.026	0.115	-0.036	0.122	0.134	-0.26	-0.183
IL-1RA	**0.656**	**0.446**	-0.197	-0.095	0.008	-0.035	-0.014	-0.055	0.192	-0.002

G-CSF	**0.585**	**-0.393**	0.177	-0.233	0.180	0.086	-0.002	-0.255	0.131	0.066
IL-4	**0.561**	**0.316**	-0.002	**0.391**	0.072	-0.119	**0.317**	0.012	-0.301	0.04
IL-10	**0.532**	**-0.434**	0.143	-0.232	-0.159	-0.144	0.019	-0.205	**0.342**	0.015
MCP-1	**0.532**	**-0.448**	0.204	0.096	0.016	-0.086	-0.121	0.218	0.000	-0.057
IL-1β	**0.456**	**0.775**	-0.149	0.034	-0.132	0.024	-0.025	-0.278	-0.019	0.043
IL-7	**0.451**	-0.178	-0.014	**0.527**	0.250	0.127	**0.419**	0.107	-0.056	-0.095
IL-5	**0.317**	-0.167	-0.199	0.246	0.273	-0.137	**-0.415**	**0.387**	0.078	0.203
IL-8	**0.302**	-0.092	0.012	0.074	0.220	**0.404**	-0.119	-0.105	0.074	**0.656**
DNMT3L	0.168	0.144	0.292	**-0.400**	-0.176	0.204	**0.358**	**0.473**	0.258	0.158
Sex	0.117	-0.026	0.012	**0.435**	**-0.390**	0.260	-0.262	0.203	0.078	-0.104
DNMT3A	0.009	0.186	**0.381**	**0.469**	-0.153	-0.162	-0.05	0.106	0.404	-0.264
LINE-1	-0.037	0.274	-0.61	-0.185	0.272	0.221	-0.054	**0.407**	0.172	-0.069
***MTHFR* C677T**	-0.058	-0.157	**-0.745**	0.235	-0.132	**0.323**	0.103	-0.076	0.193	-0.034
***MTHFR* A1298C**	-0.095	**0.311**	**0.544**	-0.021	0.248	0.166	**-0.389**	0.184	**-0.339**	0.053
DNMT1	-0.105	0.051	-0.165	0.001	-0.282	**-0.558**	0.299	0.264	-0.137	**0.485**
DNMT3B	-0.149	-0.239	0.044	**0.363**	**-0.563**	0.171	-0.106	-0.126	-0.115	**0.300**
***DHFR*_19bp**	-0.160	0.095	-0.089	0.276	**0.397**	**-0.536**	-0.176	-0.163	**0.327**	0.152
***CBS*_68bp**	-0.283	0.232	**0.539**	0.138	0.034	0.135	0.203	0.110	**0.357**	0.116
Age	**-0.356**	0.059	0.209	0.235	**0.436**	0.241	0.297	-0.222	0.027	0.074

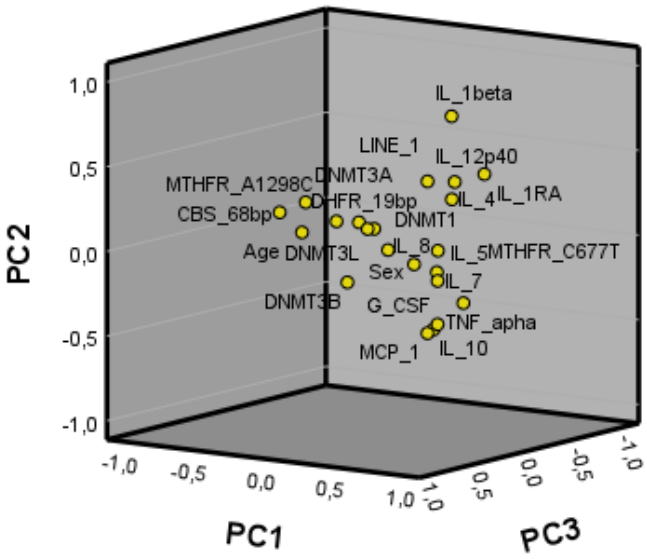


Figure 43. PCA result for the computed 22 variables of ASD group: PC1, PC2 and PC3 loadings. Distribution in the rotated space of the 22 components considering the first three PCs. Plotted by SPSS (Statistics Version 22).

The results of the analysis indicate that PC1 was predominantly made up of cytokines and age, while in PC2 a smaller group of cytokines was selected and the *MTHFR* A1298C polymorphism assumed greater weight (table 39).

Subsequently, these results were compared with the second PCA, in which comorbidities were added to the examined biochemical, genetic, and epigenetic factors. Also in this case, the adequacy of the sample was confirmed by the Kaiser-Meyer-Olkin index ($p = 0.548$) and Bartlett's test of sphericity ($p < 0.001$). Eleven components of the 28 proposed were selected, with eigenvalues between 1.046 and 4.239, explaining a cumulative variance of 73.56% (table 40).

Table 40. Total variance explained by PCA with ASD group including comorbidities

	Eigenvalues		
Component	**Total**	**% variance**	**% cumulative**
1	**4.239**	15.138	15.138
2	**2.952**	10.543	25.680
3	**2.401**	8.575	34.255
4	**2.087**	7.452	41.708
5	**1.642**	5.866	47.573
6	**1.447**	5.169	52.743
7	**1.366**	4.879	57.622
8	**1.242**	4.436	62.058
9	**1.103**	3.939	65.997
10	**1.071**	3.825	69.823
11	**1.046**	3.736	**73.559**
12	0.892	3.185	76.744
13	0.879	3.140	79.885
14	0.745	2.662	82.547
15	0.718	2.565	85.111
16	0.612	2.187	87.298
17	0.582	2.077	89.376
18	0.475	1.696	91.072
19	0.421	1.505	92.577
20	0.403	1.438	94.015
21	0.364	1.299	95.314
22	0.326	1.165	96.479
23	0.279	0.996	97.475
24	0.237	0.848	98.323
25	0.164	0.586	98.908
26	0.146	0.520	99.428
27	0.095	0.339	99.767
28	0.065	0.233	100.000

The variables selected with the greatest weight for the 11 principal components are shown in table 41, whilst in figure 44 it is shown how the 22 variables are distributed in space according to the first three PCs. The results indicate that in PC1, comorbidities take on greater significance, confirming the same cytokines as in PC2 of the analysis conducted without including comorbidities. Furthermore, the contribution of polymorphisms such as *CBS* 68bp, *DHFR* 19bp, and *DNMT3L*, as well as age, is highlighted.

Table 41. Component matrix of PCA with ASD group including comorbidities

	COMPONENT										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Lactose Intolerance	**0.702**	**0.417**	-0.025	0.117	0.133	**-0.325**	-0.011	0.042	0.097	0.134	-0.032
Gluten Intolerance	**0.693**	**0.479**	-0.050	0.131	0.012	-0.251	0.023	0.035	0.007	0.134	0.058
Intestinal Candida	**0.656**	**0.518**	0.071	0.143	-0.044	0.177	-0.031	-0.183	0.046	-0.030	-0.111
Airways Infections	**0.549**	**0.312**	0.253	0.042	0.031	0.261	**-0.516**	-0.102	0.005	-0.124	0.015
CGD	**0.455**	**0.489**	-0.192	0.035	-0.062	0.067	**-0.329**	0.140	0.195	-0.145	-0.023
Age	**0.452**	-0.059	**0.317**	-0.045	-0.215	**0.391**	**0.346**	-0.059	-0.040	0.216	-0.065
***CBS* 68bp**	**0.351**	-0.103	**0.514**	-0.141	0.196	0.095	0.000	0.053	**0.333**	0.096	0.286
***DHFR* 19bp**	**0.341**	0.160	0.006	0.270	-0.031	**-0.392**	**0.326**	0.026	**-0.367**	0.106	0.279
Epilepsy	**0.332**	-0.032	**0.587**	-0.137	**-0.413**	0.108	0.183	-0.134	-0.024	0.036	0.075
***MTHFR* A1298C**	0.126	-0.075	**0.514**	-0.099	0.137	-0.133	0.005	**0.575**	-0.072	-0.061	**-0.351**
DNMT3B	0.117	-0.027	-0.262	-0.148	**0.567**	0.162	0.029	-0.082	0.012	**0.406**	-0.152
DNMT3A	0.072	0.070	**0.334**	-0.020	**0.474**	0.021	0.218	0.004	-0.099	-0.174	**0.527**
DNMT1	0.067	-0.059	-0.210	0.178	0.222	**-0.491**	0.160	**-0.351**	**0.411**	0.031	-0.212
***MTHFR* C677T**	0.022	0.024	**-0.622**	**0.382**	-0.065	**0.435**	-0.027	-0.078	0.069	0.171	0.197
LINE-1	-0.058	-0.142	-0.215	**0.535**	**-0.434**	0.036	-0.006	**0.319**	0.264	-0.117	0.221
IL-5	-0.113	**0.425**	-0.237	0.123	-0.020	-0.182	0.201	**0.454**	0.007	-0.082	0.111
IL-8	-0.190	0.261	-0.007	-0.046	-0.102	0.128	0.062	**0.418**	0.138	**0.617**	-0.094
IL-7	-0.190	**0.528**	-0.025	0.011	0.056	**0.420**	**0.400**	-0.052	0.194	-0.178	-0.004
Sex	-0.192	0.012	-0.155	0.023	**0.598**	0.283	0.033	0.247	-0.066	-0.046	0.132
MCP-1	-0.235	**0.596**	-0.066	**-0.378**	0.049	-0.026	-0.008	0.080	0.072	-0.209	0.050
DNMT3L	**-0.320**	-0.188	0.277	-0.210	-0.035	-0.150	-0.014	0.011	**0.654**	0.101	0.220
IL-4	**-0.345**	**0.412**	0.283	**0.339**	0.136	0.064	**0.414**	-0.189	0.091	-0.048	-0.265
IL-10	**-0.365**	**0.420**	-0.099	**-0.412**	-0.045	-0.172	-0.175	-0.268	-0.099	0.214	0.272
G-CSF	**-0.398**	**0.447**	0.028	**-0.419**	-0.283	-0.025	-0.061	-0.012	-0.143	0.257	0.061
IL-1β	**-0.435**	0.072	**0.420**	**0.668**	0.107	0.022	-0.188	-0.109	-0.107	0.168	-0.069
TNF-α	**-0.489**	**0.481**	-0.043	-0.205	-0.128	-0.003	0.165	-0.042	0.027	-0.230	-0.185
IL-12(p40)	**-0.536**	**0.321**	**0.401**	0.261	0.194	-0.028	**-0.425**	0.030	-0.003	0.014	-0.031
IL-1RA	**-0.570**	0.254	0.261	**0.447**	-0.118	-0.084	-0.040	-0.094	-0.037	0.126	0.158

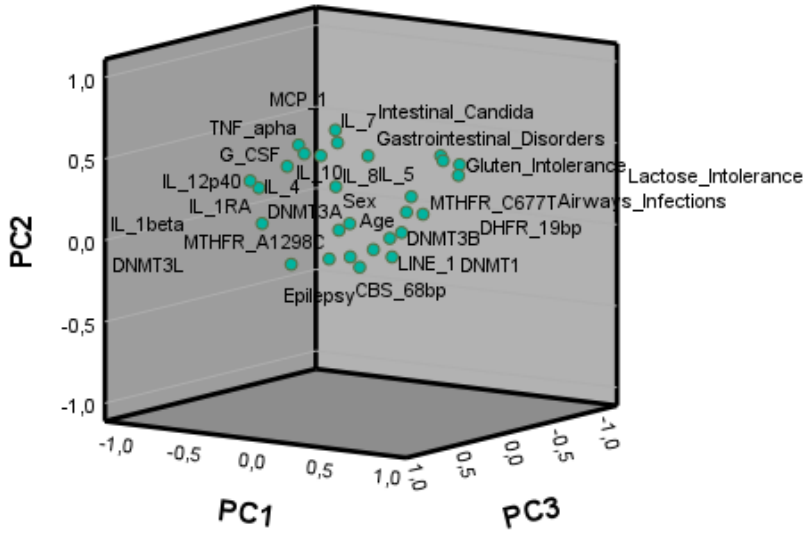


Figure 44. PCA result for the computed 22 variables of ASD group including comorbidities: PC1, PC2 and PC3 loadings. Distribution in the rotated space of the 22 components considering the first three PCs. Plotted by SPSS (Statistics Version 22).

We then used the results of these two PCAs conducted on ASD patients to perform binary logistic regression in order to identify which variables could contribute to identify differences within the ASD group, or which discriminate between patients with more severe phenotypes from those with milder symptoms among individuals with ASD.

Binary Logistic Regression in ASD group

Binary logistic regression was used to predict which biochemical, genetic, and epigenetic components might discriminate between the most severe phenotypes of ASD. PCs selected from the two PCAs conducted on the ASD group were subjected to probabilistic analysis, using the severity of the disorder as outcome. Diagnostic clinical data for ASD, such as DSM-5 manual scores, the ADOS instrument, and symptom severity, have been used to identify different levels of severity. Three regression analyses were performed, considering comorbidities, and the same results were used for further binary logistic regressions with the removal of comorbidities from the database analysed in the PCA. These analyses had two main purposes: the first was to identify the best combination of variables that predisposed to different degrees of ASD severity, while the second aimed to compare the results of logistic regressions with and without comorbidities to highlight which combination of biochemical, genetic, and epigenetic components discriminates ASD subgroups.

Below are the results of binary logistic regressions using the first 11 principal components selected by the PCA which analysed biochemical, genetic, epigenetic components along with comorbidity in the ASD group. Clinical data from DSM-5 (table 42), ADOS (table 43), and symptoms (table 44) were used as outcomes. The compared ASD subgroups consisted of patients with mild/moderate phenotype versus individuals with severe phenotype for analyses involving DSM-5 and ADOS. In contrast, for the ASD group stratified by symptoms, the two compared subgroups consisted of ASD subjects with no or minimal symptoms and patients with moderate to severe symptoms.

Table 42. Variables included in the binary logistic regression in ASD group with DSM-5 outcome including comorbidities

PCA	B	SE	Wald	df	Sign.	OR	95% CI per OR	
							Inferior	Superior
1	1.833	.537	11.672	1	**.001**	6.254	2.185	17.903
2	1.441	.462	9.711	1	**.002**	4.223	1.707	10.449
3	.713	.470	2.304	1	.129	2.041	.812	5.126
4	-.041	.448	.008	1	.928	.960	.399	2.312
5	-1.178	.464	6.435	1	**.011**	.308	.124	.765
6	.619	.444	1.938	1	.164	1.857	.777	4.437
7	.094	.396	.056	1	.813	1.098	.505	2.387
8	.233	.453	.265	1	.607	1.262	.520	3.067
9	.402	.407	.975	1	.324	1.495	.673	3.320
10	.727	.535	1.845	1	.174	2.069	.725	5.910
11	-.064	.368	.030	1	.862	.938	.456	1.931
Constant	1.158	.418	7.681	1	.006	3.183		

Statistically significant p-values ($p < 0.05$) are indicated in bold. B: Estimated regression coefficient; SE: Standard Error; Wald: statistic test; df: degree of freedom; Sign.: Significance; OR: Odd Ratio; CI: Confidence Intervals.

Table 43. Variables included in the binary logistic regression in ASD group with ADOS outcome including comorbidities

PCA	B	SE	Wald	df	Sign.	OR	95% CI per OR	
							Inferior	Superior
1	1.072	.365	8.609	1	**.003**	2.921	1.428	5.978
2	1.112	.385	8.351	1	**.004**	3.042	1.430	6.468
3	.336	.346	.944	1	.331	1.400	.710	2.758
4	.093	.309	.091	1	.763	1.098	.599	2.012
5	-.768	.367	4.387	1	**.036**	.464	.226	.952
6	.337	.374	.812	1	.368	1.401	.673	2.919

7	.272	.336	.657	1	.418	1.313	.680	2.534
8	.193	.394	.240	1	.624	1.213	.560	2.627
9	-.029	.331	.007	1	.931	.972	.508	1.861
10	.335	.411	.665	1	.415	1.398	.625	3.125
11	.267	.310	.744	1	.388	1.306	.712	2.397
Constant	1.115	.352	10.011	1	.002	3.050		

Statistically significant p-values ($p < 0.05$) are indicated in bold. B: Estimated regression coefficient; SE: Standard Error; Wald: statistic test; df: degree of freedom; Sign.: Significance; OR: Odd Ratio; CI: Confidence Intervals.

Table 44. Variables included in the binary logistic regression in ASD group with SYMPTOMS outcome including comorbidities

PCA	B	SE	Wald	df	Sign.	OR	95% CI per OR	
							Inferior	Superior
1	1.409	.437	10.384	1	**.001**	4.090	1.736	9.635
2	1.290	.417	9.566	1	**.002**	3.632	1.604	8.225
3	.528	.417	1.603	1	.205	1.696	.749	3.843
4	.014	.349	.002	1	.968	1.014	.511	2.012
5	-.412	.379	1.183	1	.277	.662	.315	1.391
6	.387	.416	.863	1	.353	1.472	.651	3.330
7	.410	.407	1.010	1	.315	1.506	.678	3.347
8	-.086	.375	.053	1	.818	.918	.440	1.912
9	.205	.345	.355	1	.552	1.228	.625	2.414
10	.405	.406	.993	1	.319	1.499	.676	3.324
11	.307	.351	.769	1	.381	1.360	.684	2.703
Constant	1.341	.411	10.662	1	.001	3.822		

Statistically significant p-values ($p < 0.05$) are indicated in bold. B: Estimated regression coefficient; SE: Standard Error; Wald: statistic test; df: degree of freedom; Sign.: Significance; OR: Odd Ratio; CI: Confidence Intervals.

Logistic regression analysis revealed statistically significant results for PC1, PC2, and PC5 when DSM-5 ($p = 0.001$, $p = 0.002$ and $p = 0.011$, respectively) and ADOS ($p = 0.003$, $p = 0.004$ and $p = 0.036$, respectively) were the outcomes, while the analysis considering symptom outcome highlighted statistically significant results for PC1 ($p = 0.001$; OR: 4.09 with CI: 1.74-9.63) and PC2 ($p = 0.002$; OR: 3.63 with CI: 1.60-8.22), figure 45. It is important to note that in the first case we compared mild/moderate phenotypes with severe phenotype, while in the second we considered ASD subjects without or with minimal symptoms compared to patients with moderate/severe phenotype. The difference in the phenotypes compared could explain the observed variation.

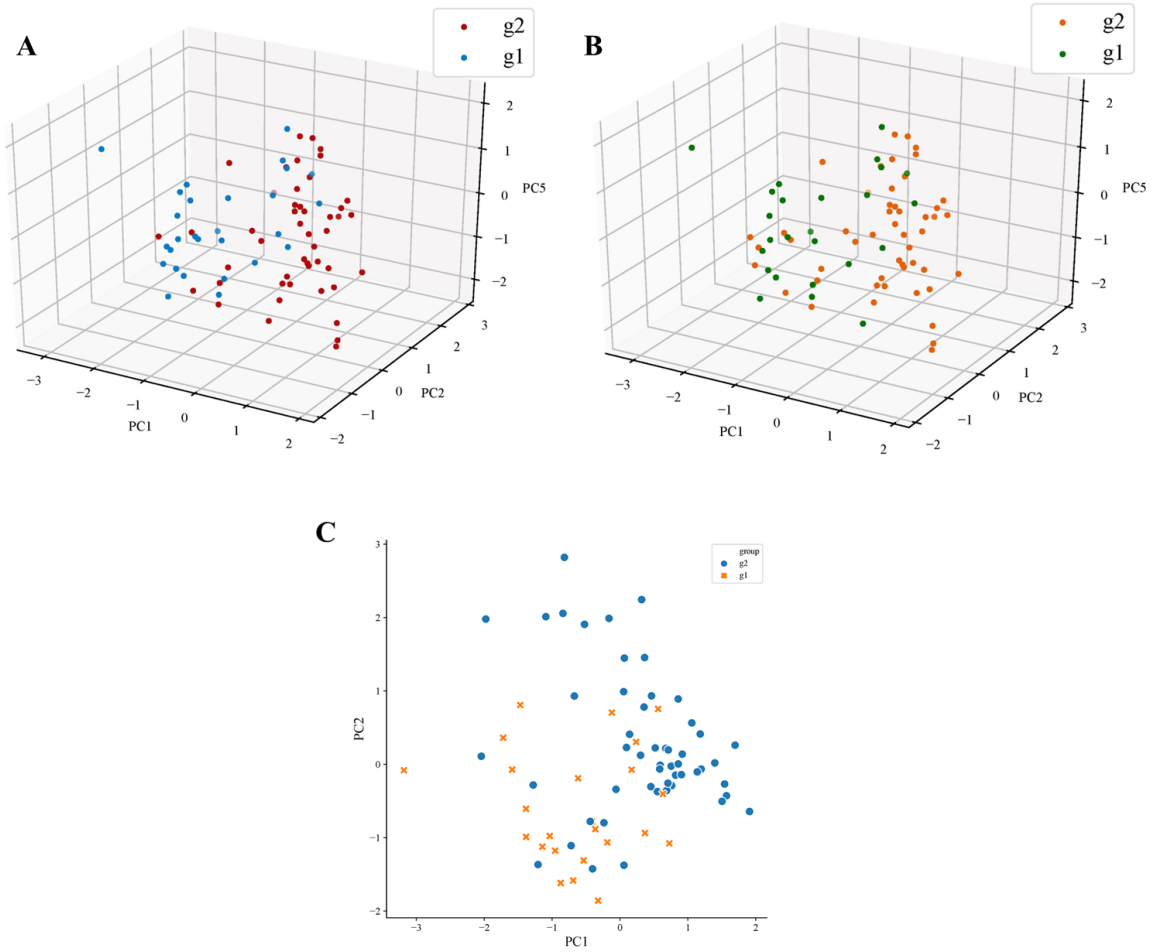


Figure 45. 3D/2D-loading plot of the ASD group score including comorbidities (n = 75) based on PC1, PC2, and PC5. (A) DSM-5 outcome, blue dots represent ASD group with mild/moderate phenotype (n=28), red dots represent ASD group with severe phenotype (n=47)**; (B)** ADOS outcome, green dots represent ASD group with mild/moderate phenotype (n=25), orange dots represent ASD group with severe phenotype (n=50); **(C)** SYMPTOMS outcome, blue dots represent ASD group with NO/minimal symptoms (n=24), orange crosses represent ASD group with moderate/severe symptoms (n=51); Plotted by bioinformatics.com.cn/srplot.

After a closer examination of the principal components identified, we observed that in PC1 comorbidities such as CGD, lactose intolerance, gluten intolerance, airway infections, epilepsy, and intestinal candidiasis were of greater importance (table 41). These comorbidities were associated with cytokines such as IL-4, IL-10, G-CSF, IL-1β, TNF-α, IL-12(p40), and IL-1RA, as well as polymorphisms in the *CBS*, *DHFR*, and *DNMT3L* genes and age. On the contrary, PC2 reduced the weight attributed to polymorphisms and age, highlighting the correlation between cytokines and comorbidities. PC5, statistically significant for DSM-5 and ADOS, consisted primarily of sex, *DNMT3B* and *DNMT3A* SNPs, *LINE-1* methylation, and epilepsy (table 41).

The obtained results were compared with regression analyses that excluded patient comorbidities to identify biochemical, genetic, and epigenetic combinations that could better discriminate patients based on the severity of ASD. Analyses using the 10 principal PCs from PCA without including comorbidities as dependent variables are reported below. Clinical data from DSM-5 (table 45), ADOS (table 46), and symptoms (table 47) were used as outcomes.

Table 45. Variables included in the binary logistic regression in ASD group with DSM-5 outcome

PCA	B	SE	Wald	df	Sign.	OR	95% CI per OR	
							Inferior	Superior
1	-.105	.306	.117	1	.732	.901	.494	1.641
2	-.340	.347	.961	1	.327	.711	.360	1.405
3	.206	.283	.530	1	.467	1.229	.705	2.141
4	.402	.309	1.687	1	.194	1.495	.815	2.741
5	1.007	.322	9.780	1	**.002**	2.737	1.456	5.145
6	.362	.339	1.145	1	.285	1.437	.740	2.790
7	-.034	.315	.011	1	.915	.967	.521	1.794
8	-.232	.295	.619	1	.431	.793	.445	1.413
9	.083	.278	.090	1	.764	1.087	.631	1.873
10	.688	.456	2.276	1	.131	1.991	.814	4.869
Constant	.748	.302	6.127	1	.013	2.112		

Statistically significant p-values ($p < 0.05$) are indicated in bold. B: Estimated regression coefficient; SE: Standard Error; Wald: statistic test; df: degree of freedom; Sign.: Significance; OR: Odd Ratio; CI: Confidence Intervals.

Table 46. Variables included in the binary logistic regression in ASD group with ADOS outcome

PCA	B	SE	Wald	df	Sign.	OR	95% CI per OR	
							Inferior	Superior
1	.041	.304	.018	1	.892	1.042	.574	1.891
2	-.344	.317	1.176	1	.278	.709	.380	1.320
3	-.008	.285	.001	1	.977	.992	.567	1.736
4	.450	.311	2.092	1	.148	1.568	.852	2.885
5	.964	.325	8.804	1	**.003**	2.621	1.387	4.953
6	.206	.332	.386	1	.534	1.229	.641	2.356
7	-.108	.337	.102	1	.750	.898	.463	1.740
8	-.077	.294	.069	1	.793	.926	.521	1.646
9	.337	.293	1.323	1	.250	1.401	.789	2.487
10	.413	.436	.900	1	.343	1.512	.643	3.553
Constant	.914	.303	9.131	1	.003	2.495		

Statistically significant p-values (p < 0.05) are indicated in bold. B: Estimated regression coefficient; SE: Standard Error; Wald: statistic test; df: degree of freedom; Sign.: Significance; OR: Odd Ratio; CI: Confidence Intervals.

Table 47. Variables included in the binary logistic regression in ASD group with SYMPTOMS outcome

PCA	B	SE	Wald	df	Sign.	OR	95% CI per OR	
							Inferior	Superior
1	-.031	.319	.009	1	.923	.970	.519	1.812
2	-.341	.333	1.044	1	.307	.711	.370	1.367
3	.241	.302	.636	1	.425	1.272	.704	2.298
4	.726	.354	4.199	1	**.040**	2.067	1.032	4.140
5	.800	.319	6.287	1	**.012**	2.226	1.191	4.162
6	.031	.343	.008	1	.927	1.032	.527	2.020
7	.185	.333	.310	1	.578	1.204	.627	2.311
8	-.136	.291	.218	1	.641	.873	.493	1.545
9	.359	.309	1.350	1	.245	1.432	.781	2.626
10	.504	.440	1.311	1	.252	1.656	.699	3.925
Constant	1.027	.321	10.213	1	.001	2.791		

Statistically significant p-values (p < 0.05) are indicated in bold. B: Estimated regression coefficient; SE: Standard Error; Wald: statistic test; df: degree of freedom; Sign.: Significance; OR: Odd Ratio; CI: Confidence Intervals.

The results of the three binary logistic regression analyses indicate that PC5 (p = 0.002, p = 0.003 and p = 0.012 for DSM-5, ADOS and symptoms, respectively) better discriminates the severity of the disorder (tables 45-47). Specifically, the variables that had the greatest weight in the analysis were sex, polymorphisms of the *DNMT3B* and *DHFR* genes, and age. Furthermore, the analysis conducted with the symptom outcome also highlighted that PC4 (p = 0.040), which consisted of cytokines such as IL-4 and IL-7, and polymorphisms of *DNMT* genes including *DNMT3A*, *DNMT3B*, and *DNMT3L*, was also able to discriminate the severity of the symptoms significantly.

Discussion

ASD is a neurodevelopmental disorder widely observed in the global population, affecting 1 child out of 100[8,9]. Epidemiological studies also indicate that ASD is diagnosed predominantly in males, with a ratio of 3:1 compared to females[10]. Indeed, in our study, the male component represented 83% of the 75 subjects diagnosed with ASD. Nowadays, the diagnosis of ASD is mainly based on clinical examinations and evaluations, while research on biochemical and genetic markers continues[3]. An important scientific challenge is precisely to identify those biochemical or genetic factors that underlie the predisposition or even the cause of ASD. The etiology of this disorder is likely multifactorial, with biochemical, genetic, and environmental factors all playing a role[2]. ASD can be divided into syndromic and non-syndromic ASD. In the first case, the disorder is often associated with monogenic alterations or chromosomal aberrations (such as Rett syndrome, fragile X syndrome, or MECP2 duplication syndrome)[2]. On the contrary, the etiology of non-syndromic ASD remains unclear. It is likely that there is a synergy of multiple factors, both genetic and prenatal and postnatal environmental[23].

To better understand the predisposition to ASD, we conducted a comprehensive study exploring biochemical, genetic, and epigenetic factors. Several scientific discoveries have identified an involvement of the immune system in ASD, but the results are still inconclusive. This could be due to the phenotypic and genetic complexity of this pathology[56]. In an attempt to identify biochemical factors underlying it, we conducted an analysis of cytokines and chemokines present in peripheral blood samples of ASD subjects and healthy siblings. The latter were selected as a control group to use biological samples from individuals of comparable age and, above all, to reduce heterogeneity and facilitate the identification of the immune bases of ASD. The analysis was conducted using a multiplex ELISA system for the simultaneous quantitative measurement of 11 inflammatory cytokines/chemokines. The results showed that IL-1RA levels were statistically lower ($p = 0.04$) in the ASD group compared to the SIBLINGS group. IL-1RA is a cytokine secreted by immune cells with antagonistic functions compared to IL-1, and which exerts a negative regulatory action on the latter[218]. IL-1RA is in fact an anti-inflammatory cytokine produced by the macrophage/monocyte lineage and by activated neutrophils, following bacterial infection. Bacterial lipopolysaccharide induces the production of pro-inflammatory cytokines such as IL-6, IL-1, and TNF-α with the aim of activating inflammation to

counteract infection. Subsequently, anti-inflammatory mediators such as IL-10 and IL-1RA come into play to extinguish inflammation[62]. The inflammatory state is strictly regulated in our bodies because if on the one hand it protects us, on the other it can cause damage if not adequately controlled. The lower levels of IL-1RA observed in individuals with ASD indicate that the inflammation does not respond in the same way as in healthy SIBLINGS.

The minimal statistical difference between the ASD group and their siblings is consistent with findings in the literature. There are numerous studies that analyse cytokine release between individuals with ASD and control groups, but few use siblings as a healthy comparison group[75,219]. When individuals with ASD are compared to healthy individuals of comparable age but unrelated, an increased systemic cytokine levels are observed, indicating an elevated inflammatory state in individuals with ASD[58]. In contrast, when the comparison of individuals with ASD is made with a group of healthy individuals of comparable age but related, i.e. their siblings, these differences vanish. A previous study demonstrated that the immunological profiles of children with ASD did not differ from those of their typically developing siblings[219]. An "autistic endophenotype" involving immune dysfunction is hypothesized among family members who are apparently unaffected by the main symptoms of ASD[220]. Children with autism and unaffected siblings share the presence of anti-neuronal antibodies. Furthermore, healthy siblings show mild neurological impairment and there is evidence of atypical communicative and social development during childhood[221]. It is possible that a similar immunological background to that of siblings could lead to different clinical outcomes, i.e. the onset of the disorder, when fetal development is exposed to environmental stressors such as bacterial or viral infections, or prenatal exposure to environmental toxins[222].

To further investigate the immune response in ASD, we correlated cytokine levels with various clinical traits of the patients. It is well documented in the literature that ASD subgroups may differ in their immune response. The analysis allowed us to identify some cytokines as predisposing factors for more severe phenotypes of the disorder. For the stratification of the ASD group, key clinical characteristics such as the DSM-5 manual score, the ADOS diagnostic tool, the symptom severity, the presence of OCD, intellectual disability, and the presence of aggressive behaviors were considered. Investigating the differential immune response in ASD subgroups could help identify potential therapeutic targets to reduce the most severe manifestations of the disorder, thereby alleviating symptoms and improving patients' quality of life.

Our results showed that IL-1β levels were statistically higher in the severe phenotype of patients compared to the moderate phenotype when the ASD group was stratified based on two key clinical data: DSM-5 score ($p = 0.025$) and intellectual disability ($p = 0.049$). Unlike DSM-5 and IQ, the analysis of patient stratification by symptom severity did not show statistically significantly higher IL-1β levels but rather a tendency to increase in patients with moderate/severe symptoms compared to individuals with none or minimal symptoms. This discrepancy may be due to the small number of patients with severe symptoms recruited in this study.

The involvement of IL-1β in the pathophysiology of ASD is supported by several studies that have recorded increased levels of the leukocyte pyrogen in the plasma of ASD children[62,218]. IL-1β is expressed early in immune responses and, in tissues, propagates inflammation by activating local immune cells, while at a systemic level, it stimulates the production of IL-6[223]. IL-1β can cross the blood-brain barrier, and its receptors are present throughout the central nervous system. In the hippocampus, it stimulates its own production to trigger neuroendocrine changes associated with fever. Furthermore, it plays a crucial role during development, modulating the proliferation of neuronal progenitor cells in various regions of the central nervous system. Disruption of this modulatory function could potentially explain the impaired growth observed in the brains of individuals with ASD[218]. IL-1β is also implicated in the formation of excitatory synapses. Therefore, an alteration of IL-1 family cytokines and their receptors could disrupt the balance between excitatory and inhibitory signalling of neurons, which could influence the neurological characteristics of ASD. To regulate the action of IL-1β, immune cells produce IL-1RA, which dampens inflammation by competing for binding to the receptor[218]. Increased levels of IL-1RA have been recorded in individuals with ASD, suggesting an attempt to counterbalance IL-1β by counteracting inflammation[59]. Analysis of the ASD group stratified by ADOS showed increased levels of IL-1RA in subjects with severe ASD phenotype compared to those with mild/moderate phenotype ($p = 0.059$). The same group also exhibited a slight increase in IL-1β levels, which could explain the observed increase in IL-1RA.

From our intra-case analyses, we also observed changes in systemic levels of TNF-α, a pro-inflammatory cytokine capable of crossing the blood-brain barrier like IL-1β. Results indicated that TNF-α levels were statistically reduced in individuals with severe ASD phenotype following stratification by DSM-5, OCD, and aggressive behaviour ($p = 0.01$ and $p = 0.03$; $p = 0.03$; $p = 0.048$, respectively). Numerous studies in the literature have

demonstrated increased levels of TNF-α in individuals with ASD compared to healthy controls[67,224], but only few papers have shown changes in the levels of the pro-inflammatory cytokine after stratifying individuals with ASD based on symptom severity[72]. In one of these, they demonstrate an increase in TNF-α concentration in the blood of subjects with severe symptoms due to the reduced expression of the THRIL gene involved in its regulation[225]. In contrast, in another study, low levels of TNF-α were associated with patients with irritability. Furthermore, previous observations have correlated a decrease in immune markers, in particular Ig levels, with behavioural symptoms in ASD[226].

In addition to TNF-α, IL-12(p40) levels were also statistically lower in the ASD subgroup with severe aggressive behaviors compared to subjects without aggression ($p = 0.048$ and $p = 0.026$, respectively). The function of IL-12(p40) is not well understood, although it has been identified as a component of the cytokines IL-12 and IL-23. Only recently, it has been demonstrated that it has an autonomous function as a chemotactic cytokine for macrophages and is implicated in stimulating the migration of bacteriologically activated dendritic cells[227,228]. Increased levels of IL-12(p40) have been associated with clinical phenotypes of ASD such as hyperactivity and stereotyped behaviors, but no data on the aggressive phenotype of patients are reported[56,229].

Chemokines analysis in plasma samples from the ASD group revealed that only G-CSF and MCP-1 were detectable. These two cytokines showed higher levels in the plasma samples of the ASD subgroup with impaired intellectual abilities compared to patients with normal IQ ($p = 0.052$ and $p = 0.016$, respectively). Several cytokines were inversely correlated with IQ, including IL-1β, IL-6, IL-10, and MCP-1, in autistic children with sleep problems and aggressive behaviors[56,230]. These data support the increased IL-1β levels observed in the ASD subgroup with intellectual disabilities. Furthermore, a positive correlation has been observed between social dysfunction in ASD individuals and cytokines such as MCP-1, IL-1β, MIP-1, and GM-CSF[56].

Increased levels of IL-1β, IL-6, and MCP-1 have also been associated with alterations in cognitive and adaptive functions in patients with ASD[75]. Other studies have found increased levels of MCP-1 in brain tissues and cerebrospinal fluid, along with marked activation of microglia and astrocytes in autistic patients[52].

It is plausible to hypothesize that individuals with ASD experience dysfunction of the inflammatory system, leading to an overproduction of pro-inflammatory cytokines such as IL-1β and chemokines such as G-CSF and MCP-1. This hyperactivation may be triggered by various factors, including oxidative stress, viral and bacterial infections, or other

environmental stimuli. Research suggests that ASD patients may exhibit compromised blood-brain barrier function, which regulates the passage of substances between the bloodstream and the brain. This compromise could allow peripheral immune system-produced cytokines to penetrate the brain uncontrollably, thus contributing to brain inflammation. Activation of immune cells such as microglia and astrocytes has been observed in the brains of ASD subjects. These immune components can, in turn, secrete cytokines, including G-CSF, MCP-1, and IL-1β, in response to inflammatory stimuli, thereby contributing to brain inflammation that may influence the intellectual deficits observed in individuals with ASD.

Patients with ASD, in addition to the characteristic symptoms of the disease, suffer from numerous comorbid conditions that influence the course of the disease itself. The immune system in ASD is considered an important component that contributes not only to the pathogenesis of the disorder or to explaining the different clinical phenotypes, but could also help to identify how comorbidities influence the severity of ASD[56,231]. For this reason, we investigated the contribution of the inflammatory system to the comorbidities of patients with ASD. Epilepsy and gastrointestinal disorders are common comorbidities in ASD, especially in individuals with intellectual disabilities[232]. In the examined ASD group, many individuals suffered from CGD and intestinal infections, but only a few had seizure events. The comorbidities that we considered in the analysis of cytokine release were epilepsy, upper respiratory tract infections, CGD, intestinal candidiasis, and lactose and gluten intolerance. From analyses of the ASD group stratified by each comorbidity, we observed several correlations between cytokine production and comorbidity. In the first result, we observed decreased IL-5 levels in patients with epilepsy compared to ASD individuals without epileptic events ($p = 0.011$). The reduced production of anti-inflammatory cytokines such as IL-5 can compromise the regulation of inflammation, particularly the inhibition of inflammatory responses. The decrease in its levels could contribute to the establishment of a chronic inflammatory state in the brain and central nervous system, which may influence ASD symptoms and the onset of epilepsy.

Continuing the analyses of comorbidities in patients, we observed reduced levels of IL-8 in ASD individuals with lactose intolerance compared to the subgroup without intolerance.

These data are preliminary and require further investigation since the two ASD subgroups showed a numerical disparity.

An interesting result was observed in the ASD subgroup stratified by intestinal infections, particularly candidiasis. Patients with intestinal candidiasis showed statistically lower levels

of G-CSF, IL-1RA, and IL-8 compared to patients who had never suffered from intestinal infections. Furthermore, these three cytokines tended to increase in plasma samples in the ASD subgroup experiencing constant infections compared to ASD individuals with sporadic events ($p = 0.18$, $p = 0.017$ and $p = 0.039$, respectively). The cytokine levels in ASD subjects with chronic infections were comparable to those in patients who had never suffered from intestinal infections ($p = 0.023$, $p = 0.030$ and $p = 0.051$, respectively). This observation may be consistent with the reduced inflammatory response during a chronic infection. Prolonged infection could lead to minor variations in cytokine levels compared to sporadic or discontinuous infections. This situation may arise from an adaptation of the immune system to the constant presence of the pathogen or from an immunological regulatory mechanism to keep inflammation under control and prevent damage to host tissues.

Candida is normally present in our body, but when it overgrows, it can alter the intestinal balance and the composition of the microbiota, leading to clinical symptoms such as constipation, diarrhoea, bloating, and intestinal pain. The gut microbiota plays a crucial regulatory role in maintaining the body's homeostasis. Indeed, the gut-brain-microbiota axis is studied in psychiatric and neurodegenerative disorders to clarify its functioning and identify therapeutic strategies for neuropsychiatric disorders[54]. The microbiota and the brain communicate through various pathways such as the immune system and the enteric nervous system, influencing brain development and behaviors[54]. Many factors can influence the gut microbiota in the first years of life, such as infections, antibiotic use, nutrition, environmental stress, and host genetics. Recent studies have associated the gut microbiota with diseases including ASD, schizophrenia, Parkinson's and Alzheimer's disease. Several studies have highlighted alterations in the composition of the microbiota in children with ASD[233]. Furthermore, it has been observed that patients treated with broad-spectrum antibiotics, such as vancomycin, showed a significant improvement in behavioural symptoms[234].

What has been reported so far constitutes preliminary evidence that has sparked interest in understanding the biology and physiology underlying the brain-gut-microbiota axis and become a therapeutic target for disorders such as ASD[235].

The reduction of anti-inflammatory cytokines such as IL-1RA, as well as pro-inflammatory cytokines like IL-8 and G-CSF, observed in ASD patients with intestinal infections, could be the result of inflammatory system dysfunction caused by alterations in the intestinal microbiota. Imbalances in the microbiota can influence cytokine production systemically and may compromise communication between the gut and the brain, thereby exacerbating clinical manifestations in patients with ASD.

Our analyses have demonstrated the existence of inflammatory system dysregulation correlated with more severe ASD phenotypes. Such evidence should be further explored by increasing the number of cases analysed and including a group of individuals not affected by ASD but with clinical manifestations of the most common comorbidities, such as gastrointestinal disorders.

Since ASD is recognized as a multifactorial disorder, we decided to also study epigenetic and genetic factors, in addition to the inflammatory component, as predisposing factors for ASD. In particular, we conducted analyses on the degree of global DNA methylation in whole blood samples from the ASD and SIBLINGS groups. This epigenetic mechanism influences gene expression and genome stability and can be altered by environmental factors. As a marker of global DNA methylation, we selected the *LINE-1* sequence, specifically the degree of methylation of CpG sites in the *LINE-1* promoter. Epigenetic mechanisms are essential because they influence the progression of different developmental stages. Furthermore, alterations in *LINE-1* methylation have been correlated with neural tube defects and specific ASD phenotypic groups, such as individuals with the CHD8 variant, where global *LINE-1* and *Alu* methylation were significantly reduced[204]. On the contrary, no statistically significant differences emerged from the comparison of methylation levels between the ASD group and the control group ($p = 0.40$). In the present study, we analysed *LINE-1* methylation between patients with ASD and unaffected siblings to confirm that there were also no differences with the SIBLINGS group. Autism is highly heterogeneous from both clinical and biological perspectives. This means that ASD patients may exhibit a wide range of symptoms and characteristics, which can also be reflected in epigenetic modifications such as DNA methylation. The lack of significant differences could be attributed to the variety of ASD subtypes present in the study, which may have different methylation profiles.

Once this hypothesis was confirmed, we further analysed the stratification of the ASD and SIBLINGS groups for the main polymorphisms of the one-carbon cycle. 8 polymorphisms belonging to 7 genes were selected for stratification, including *MTHFR* C677T rs1801133, *MTHFR* A1298C rs1801131, *DHFR* 19bp rs70991108, *CBS* 68bp rs876657421, *DNMT1* rs2228612, *DNMT3A* rs734693, *DNMT3B* rs1569686, and *DNMT3L* rs2070565. The first 4 polymorphisms are genes implicated in the folate cycle and have been associated with neurodevelopmental disorders such as ASD[32]. This cycle is directly linked to the methylation mechanism in which methyl groups are transferred to methyl acceptors, namely the DNMTs, to be then utilized in the epigenetic mechanism of methylation. The 4 selected *DNMT*

polymorphisms belong to the set of genes that directly participate in the modulation of the global DNA methylation level. From the comparison analysis between the ASD and the SIBLINGS groups, we observed that the TT genotype of the *MTHFR* C677T rs1801133 polymorphism had a higher *LINE-1* methylation percentage compared to siblings with the same genotype ($p = 0.055$). Furthermore, *LINE-1* methylation increased with increasing T allele in the ASD group. On the contrary, analysis of the ASD and SIBLLINGS groups stratified by the rs1801131 SNP showed a tendency towards a decrease in the *LINE-1* methylation percentage in the CC genotype compared to the AA genotype in both groups examined.

In the literature, it is reported that both transition and transversion polymorphisms of the *MTHFR* gene reduce the enzyme activity[128], these data are in line with the results of the analysis of *MTHFR* A1298T. In contrast, individuals with the TT genotype for *MTHFR* C677T did not show lower levels of *LINE-1* methylation compared to subjects with the CC genotype. It should be remembered that the efficiency of the one-carbon cycle is influenced both by environmental factors such as folate intake during pregnancy and by genetic factors such as the other molecular components of this complex cycle.

In the next step, we stratified the population of patients and unaffected siblings for *DNMT* gene polymorphisms. An interesting finding was obtained by stratifying for *DNMT3B* rs1569686, where ASD individuals with GT and TT genotypes had lower *LINE-1* methylation levels than ASD patients with GG genotype ($p = 0.049$ and $p = 0.15$, respectively). This result was also confirmed analysing the ASD group in the dominant model, where subjects with GG genotype showed a statistically significantly higher *LINE-1* methylation percentage than patients with GT and TT genotypes ($p = 0.040$). Our analyses confirm that other genetic factors such as the *DNMT3B* SNP gene influence the degree of *LINE-1* methylation in the ASD group. The rs1569689 polymorphism, being located in a regulatory region of the gene, could lead to altered efficiency of gene expression and therefore of the downstream enzyme with a consequent decrease in the level of global DNA methylation.

Since the ASD group is heterogeneous, we stratified patients based on clinical data to identify possible differences in global DNA methylation between subgroups of ASD individuals. Indeed, analyses showed a difference in *LINE-1* methylation levels in the group stratified by DSM-5 and intellectual disability. Patients in the subgroup with a moderate phenotype showed borderline lower levels of transposon methylation than individuals with

a mild ASD phenotype, when stratified by DSM-5 ($p = 0.055$). Furthermore, patients with severe intellectual disabilities had statistically lower levels of *LINE-1* methylation than patients with moderately reduced IQ ($p = 0.0037$). The reduction in *LINE-1* methylation in severe phenotypes reflects the genomic instability of these patients compared to individuals with milder ASD phenotypes and identifies the influence of an epigenetic component in more severe clinical phenotypes. This suggests that DNA methylation may be involved in the progression of symptom severity in ASD. The reduction in *LINE-1* methylation could be associated with increased susceptibility to genomic mutations or genetic instability, which may contribute to the severity of symptoms in ASD. Furthermore, it suggests that epigenetic changes, such as DNA methylation, may represent an important risk factor in the manifestation of more severe symptoms in ASD. These findings underscore the importance of better understanding the role of epigenetics in the pathogenesis and severity of ASD symptoms in order to develop targeted therapeutic strategies.

Next, we investigated the degree of correlation of *LINE-1* methylation between children and parents to identify subtle differences in the inheritance of methylation information between parents and affected and unaffected children. As expected, the degree of correlation was high for both parents compared to both groups of children. In the mother-child correlation, however, we observed a stronger correlation with affected children than with healthy children a difference not observed in the father-child comparison. These preliminary data could be further investigated by examining the methylation of ASD-related gene regions.

To better analyse a multifactorial disorder such as ASD, we used a multivariate analysis approach. We performed PCA and binary logistic regression analyses on experimental and clinical data to identify a combination of multiple variables as possible diagnostic and prognostic markers in ASD. In the initial investigation, we considered biochemical, genetic, and epigenetic components of both the ASD and SIBLINGS groups to see if there were differences between them that single analyses had not shown. Principal Components regression analysis showed that PC6 and PC7 can better discriminate between the ASD and SIBLINGS groups. We found a significant positive association with ASD risk in PC6 and an overall negative association with ASD risk in PC7. PC6 was characterized by associations between *DNMT3A*, *DNMT1*, *DHFR* 19bp, and sex, while PC7 was characterized by a strong association between *DNMT3L*, *DNMT3B*, and *CBS* 68bp. This result highlights that there is no difference between affected and unaffected siblings when analysing cytokine levels,

which is consistent with the small statistical difference observed between the two groups in single analyses, instead emphasizing a difference in genetic factors.

Shifting the focus of the study to an intra-case view, we performed PCA and binary regression analyses on the ASD group, using biochemical, genetic, and epigenetic factors as variables, and subsequently in association with clinical data. Considering comorbidities in the analysis, logistic regression showed that PC1, PC2, and PC5 discriminated between different phenotypic levels of patients, considering DSM-5 and ADOS. In contrast, for symptoms, only PC1 and PC2 were significant. In particular, PC1 and PC2 showed a positive association with ASD severity and were more characterized by the relationship between comorbidities and cytokines. In addition, PC1 also showed a strong association with polymorphisms of the *DNMT3L, DHFR, CBS* genes and age. In contrast, PC5 showed a negative association for severe phenotypes and was mainly composed of sex, *DNMT3B* and *DNMT3A* SNPs, *LINE-1* methylation, and epilepsy. When comorbidities were removed from the analysis because they are closely related to ASD, we observed that PC5 better discriminated the severity of the disorder. In particular, the variables that had the most weight in the analysis were sex, gene polymorphisms of *DNMT3B* and *DHFR*, and age. Furthermore, the analysis conducted with symptom outcomes also highlighted PC4, which consisted of cytokines such as IL-4 and IL-7, and *DNMT* gene polymorphisms such as *DNMT3A*, *DNMT3B*, and *DNMT3L*.

Conclusion, limitations and future prospects

The results obtained in this study confirmed the multifactorial component that predisposes to ASD. We can validate the significant role of the inflammatory system, especially the alteration of systemic cytokine release, in ASD patients. This study is one of the few that considers healthy and related individuals as a control group. The comparison between the patients and the Siblings groups showed similar levels of cytokines/chemokines, highlighting the alteration in the levels of an anti-inflammatory cytokine, IL-1RA, reduced in the patient group.

We observed alterations in systemic cytokine levels and genetic and epigenetic factors when key clinical characteristics and comorbidities of ASD subjects are examined. The study highlights the importance of identifying and characterizing ASD subgroups to identify phenotype-specific biomarkers useful for implementing personalized therapeutic strategies. Intra-case analyses showed altered cytokine release, such as increased levels of IL-1β, IL-1RA, G-CSF, and MCP-1, and decreased TNF-α and IL-12(p40) in individuals with severe ASD phenotypes. Interesting data were obtained considering patient comorbidities such as epileptic events and intestinal infections. Overall, the data indicate that more severe ASD phenotypes are related to impaired regulation of the inflammatory system.

Global DNA methylation analyses showed similar *LINE-1* methylation levels between individuals with ASD and healthy siblings. The close correlation of methylation was confirmed between affected and unaffected children and their parents. However, alterations in *LINE-1* methylation were observed between the ASD and SIBLINGS groups after stratifying for the *MTHFR* C677T (rs1801133) SNP. In addition, the ASD group showed altered *LINE-1* methylation levels as a function of stratification for the SNP of the *DNMT3B* gene (rs1569686). These results show that the ASD group presents alterations in the methylation mechanism associated with genetic factors implicated in the one-carbon cycle.

Furthermore, *LINE-1* methylation alterations observed in severe ASD phenotypes indicating impaired genomic instability in specific subgroups.

The multivariate approach highlighted that the difference between the ASD group and the SIBLINGS group lies in the combination of genetic factors, such as polymorphisms of the *DNMT, DHFR* and *CBS* genes, and sex, rather than in biochemical factors such as cytokines. In contrast, intra-case analyses revealed the association between cytokines and comorbidities

and between *LINE-1* methylation and genetic factors such as and polymorphisms such as *DNMT3A, DNMT3B* and sex.

The results obtained in this study will need to be further confirmed by increasing the number of ASD and sibling samples. It may also be necessary to include a control group of healthy, age-matched, but unrelated individuals in the analysis to compare both the patient and healthy sibling groups. To further explore the role of the inflammatory system, subjects without a diagnosis of ASD but with the same comorbidities such as patients' gastrointestinal disorders, could be included to obtain more personalized results.

Deeper investigation is needed for epigenetic mechanisms, such as exploring other global DNA methylation biomarkers such as *Alu* and *SAT*. Investigating the methylation of promoters of gene related to the immune system, the folate cycle, or the methylation mechanism could provide valuable insights. The one-carbon cycle has many molecular players involved in the reactions, so it may be justified to consider other genes as candidates for ASD susceptibility. Similarly, significant cytokine polymorphisms could also be added to the analyses.

The environmental component must not be overlooked as a predisposing factor for ASD. Therefore, it is necessary to investigate the nutritional and health status of mothers during pregnancy. This information could help to better understand the difference between ASD subjects and healthy siblings and the severity of the ASD phenotype.

In this study, we identified potential genetic factors involved in ASD by analysing targeted polymorphisms in the carbon cycle or methylation pathways. An interesting and valid approach that could contribute to a comprehensive understanding of the genetics underlying ASD is the combined use of two tools: Whole Exome Sequencing (WES) and Genome-Wide Association Studies (GWAS). By combining WES and GWAS, it is possible to identify new targets for personalized treatments and improve current therapies by discovering rare mutations and common variants. Furthermore, these techniques help to unravel the biological mechanisms underlying ASD, such as neuronal signalling pathways and neuroplasticity, contributing to the development of new early intervention and prevention strategies.

The results obtained and further investigations could help characterize ASD patients to identify useful biomarkers to implement personalized therapies or a combination of prognostic molecular markers for the broad spectrum of the disorder.

Abbreviation

5-methyl-THF	5-methyltetrahydrofolate
ADDM	Autism and Developmental Disabilities Monitoring Network
ADHD	Attention Deficit Hyperactivity Disorder
ADI-R	Autism Diagnostic Interview - Revised
AdoHcy	S-adenosylhomocysteine
AdoMet	S-adenosylmethionine
ADOS-2	Autism Diagnostic Observation Schedule - II edition
APA	American Psychiatric Association
AS	Asperger's Syndrome
ASD	Autism Spectrum Disorders
BH4	Tetrahydrobiopterin
BHMT	Betaine Homocysteine Methyltransferase
CBS	Cystathionine β-synthase
CDC	Centers for Disease Control and Prevention
CGD	Chronic Gastrointestinal Disorders
CNV	Copy Number Variants
CTH	Cystathionase γ-synthase
dATPαS	Deoxyadenosine α Theotriphosphate
DHF	Dihydrofolate
DHFR	Dihydrofolate Reductase
DNMT	DNA methyltransferase
DNMTs	DNA methyltransferases
dNTPs	Deoxynucleoside Triphosphates
DSM-5	Diagnostic and Statistical Manual of Mental Disorders - Fifth Edition
FR	Folate Receptors
FRET	Fluorescence Resonance Energy Transfer
G-CSF	Granulocyte-Colony Stimulating Factor
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor

GRIK2/GluR6	Glutamate Receptor 6 gene of the Kainate Receptor Subunit
Hcy	Homocysteine
IFN-γ	Interferon-γ
IL-	Interleukin-
IQ	Intellective Quotient
LINE-1	*Long Interspersed Element-1*
MAT	Methionine Adenosyltransferas
MCP-1	Monocyte Chemoattractant Protein-1
MeCP2	Methyl-CpG Binding Protein 2
MGB	Minor Groove Binder
MIP	Macrophage inflammatory protein
MTHFD	Methylene-THF Dehydrogenase
MTHFR	Methylene Tetrahydrofolate Reductase
MTR	Methionine Synthase
MTRR	Methionine synthase Reductase
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NFQ	Nonfluorescent Quenchers
NMDA	N-methyl-D-aspartate
non-LTR	non-long terminal repeat
OCD	Obsessive-Compulsive Disorder
OR	Odd Ratio
ORF	Open Reading Frame
PCA	Principal Component Analysis
PCFT	Proton-Coupled Folate Transporter
PCR	Polymerase Chain Reaction
PCs	Principal Components
PDD-NOS	Pervasive Developmental Disorder - Not Otherwise Specified
PPi	Pyrophosphate
PPP	Platelet-Poor Plasma
RFC	Reduced Folate Carrier

RT	Room temperature
SAHH	S-adenosylhomocysteine hydrolase
SAM	S-adenosyl-methionine
SD	Standard Deviations
SHMT	Serine Hydroxymethyltransferase
SNP	Single Nucleotide Polymorphisms
TBE	Tris Borate EDTA
THF	Tetrahydrofolate
TNF-α	Tumor Necrosis Factor-α
TYMS	Thymidylate Synthase

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