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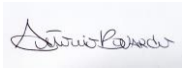
Metabolomics features associated to microbiota alterations in autism spectrum disorder

PhD student

Antonio Belardo



Coordinator of PhD course

Prof. Claudio Carere

Tutor

Prof.ssa Sara Rinalducci

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Summary

Aims

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by severe disturbance in socialization, qualitative communication impairment, and repetitive or unusual behavior.

ASD is increasingly recognized as a public health problem, as cases of children with this disease have increased sharply. Improvements in diagnostic criteria probably contributed to this obvious increase. Several studies have shown the presence of alterations in neuropsychological function and learning disabilities in subjects affected by alterations in metabolism, who demonstrated a progressive neuropsychological decline in different areas of the intellect, attention, memory and executive functions. Recently, some metabolic abnormalities have also been related with various diseases involving the behavioral disorders associated with autism. Although autism spectrum disorder is still diagnosed through behavioral observation due to the lack of biomarkers, recent metabolomics studies on human biofluids have provided a sensitive tool for identifying metabolite profiles that may greatly help clinicians offer more accurate and reliable diagnoses. Given the complexity of the genetic environment of ASD, metabolomic profiling can provide an alternative pathway for the development of early diagnostic tests. Many investigations have been conducted using metabolomics, which aims to identify the global biochemical signature of autistic individuals, in an attempt to determine the perturbations of specific metabolic pathways related to this condition.

In this thesis I tried to identify, through metabolomic analyses on urine samples, the altered metabolites that could be common in a large number of autistic children and then to study in detail the metabolic pathways connected, first to neurotransmission, and then to vitamin B6, vitamin B12 and folic acid (vitamin B9).

Vitamins, minerals and essential amino acids are by definition fundamental to human health, especially due to their critical function as enzyme cofactors for numerous reactions in the body, such as the production of neurotransmitters and the metabolism of fatty acids. For some time, attention has been focused on inadequate intake of vitamins and minerals, mainly due to a diet poor in these elements, as the main factor contributing to many health

problems. However, nutritional status depends not only on intake, but also on digestion, absorption, transformation and metabolic demand. The B vitamins are involved in the regular development of metabolic pathways (in particular transulfuration and transmethylation) necessary for the cell for its activity and survival. The lack of absorption and presence of these compounds can link to intestinal dysbiosis already found in autistic patients. Furthermore, intestinal dysbiosis also has a negative effect on neurotransmission. The metabolites produced by the altered microbiome can block specific metabolic pathways, resulting in a greater or lesser presence of certain neurotransmitters. All of this manifests itself in the ASD phenotype.

The analyses carried out in this thesis confirmed the key role that the microbiome plays on the pathogenesis of autism. A deficit of B vitamins, malabsorbed due to the microbiome, may cause hypomethylation in autistic children. Microbiome contributes to the alteration of metabolism by causing an incorrect absorption of essential substances for the human body and producing harmful metabolites especially in the brain.

1. Autism spectrum disorder

Autism Spectrum Disorder (ASD) is a phenomenon that was studied and conceptualized in the 20th century. Until then, past cases of autism were classified as mental health disorders or intellectual disabilities (Cuxart, 2000). However, some behavioral descriptions indicating the existence of ASD are documented, hinting at cases of ASD in history (Alonso, 2009). Among the few examples, we can find the notes written by the apothecary of the London asylum on a five-year-old boy hospitalized in 1799 (Haslam, 1809). In 1925, psychiatrist Grunya Sukhareva described many of the characteristics of autism in six children diagnosed with "schizoid personality disorder in childhood" (Alonso, 2009; Feinstein, 2016; Sukhareva, 1925). In 1943 the psychiatrist Leo Kanner and in 1944 the pediatrician Hans Asperger both independently conducted similar work and published it a few months apart (Cuxart, 2000). They discovered a disorder that led to extremely specific problems and various alterations and called it "autism" (Asperger, 1944; Kanner, 1943).

Kanner and Asperger treated children who shared many characteristics, but who at the same time had some differences. These differences became the basis for two different syndromes, that of Kanner and that of Asperger's. Kanner proposed a new clinical category, early childhood autism, and found that this was more prevalent in boys, described as a triad of deficiencies characterized by specific behavioral signs, impairments in socialization and communication. Despite the inter-individual differences, those children had common characteristics: (a) loneliness; (b) a desire for the maintenance of equality; (c) concern for particular objects; (d) muteness or language not used for communication; and (e) islets of skill (Kanner, 1943). On the other hand, Asperger's syndrome (1944) referred to children who had a developmental disorder but with good intellectual and linguistic abilities. Children, despite having abnormal social behaviors, strange obsessions and a preference for routine, may be intellectually bright and display highly developed language (Cuxart, 2000; Silberman, 2015).

In 1980 the American Psychiatric Association (APA) recognized autism as a condition other than schizophrenia in the third edition of the "Diagnostic and Statistical Manual of Mental Disorders" (DSM-III; APA, 1980). In the following DSM-IV (APA, 1994) and

DSM-IV-TR (APA, 2000), autism and associated disorders have been defined as "pervasive developmental disorder" (PDD), a general category that combines five diagnoses, all based on the triad of autism suggested by Wing (1979): autistic disorder, Asperger's syndrome, Rett's syndrome, childhood disintegrative disorder and PDD-NOS (not otherwise specified). The new Diagnostic and Statistical Manual of Mental Disorders (DSM-V) (American Psychiatric Association, 2013), on the other hand, introduced the following definition of autism spectrum disorders: given the wide range of symptoms characteristic of the disease and the varying degrees of severity, what we generally call autism is now referred to by the scientific community as "Autism Spectrum Disorders" (ASD) and includes the different subtypes of PDD, with the exception of Rett syndrome (Balmaña and Calvo, 2014), which was removed from this clinical category because its diagnosis is based on genetics. The new DSM-5 classifications reflect a phenotypic variability of the disease in two dimensions, so that the triad of symptoms has been reduced to a dyad characterized by the presence of (a) deficiency in social interaction and communication and (b) limited and repetitive behaviors and interests.

At present, autism encompasses a spectrum of disorders and autistic individuals are classified according to different types of abilities that depend on their individual intellectual and social development. There are several theories that attempt to explain the difficulties caused by autism; one of these, called "theory of mind", lists some typical deficits caused by autism and emphasizes how autistic patients have difficulty in interpreting and interacting with the environment and surrounding people (Glazebrook et al., 2008). Compared to "unaffected" children, these difficulties can cause a lack of understanding of one's own intentions and of others. The true mechanisms involved in autism are not yet fully understood, but are believed to be related to abnormal neurotransmission and malfunctions of specific regions of the brain, in particular the limbic system, the amygdala region, the cerebellum and other areas of the brain such as sub-corticals that mediate motor control (Bauman et al., 2005). Due to the marked impairment of mental functions and difficulties in social interaction, there is a need to find non-invasive techniques that could help in the early diagnosis of autism. In fact, studies on the Kanner triad (Kanner, 1943) do not focus on the early development of children when in reality some of the most common symptoms are evident already in the first years of life and include difficulties in sensory processing (Baranek, 2002; Dawson and Watling, 2000), limited social interaction (Gevers

et al., 2006), deficits or delays in language development (Smith et al., 2007), and unusual or problematic behaviors (Horner et al., 2002). The symptoms of autism almost always appear in the first three years of childhood and the first obvious difficulties concern the development of social relationships and language (the most common mistake is for example the use of "you" instead of " I "or vice versa, given by a difficulty in distinguishing people), as well as the presence of repetitive and compulsive behaviors as rituals and largely mental retardation.

Given the difficulties in formulating an accurate prognosis for autism, a test is often performed to evaluate the IQ. As a result, children with a quotient below 50 are almost always autistic. This test is based on the words that a child of about 7 can use. Furthermore, more than 75% of affected subjects present, albeit in a mild form, mental retardation (Smalley, 1997). The severity of the symptoms of autism varies a lot and in most cases tends to improve with age, even if total recovery is practically impossible, as it would appear to be a genetic deficiency.

Several decades ago, epidemiological research studies estimated that the incidence of ASD was in a ratio of about 4 to 5 cases per 10,000 people (Lotter, 1966; Ritvo et al., 1989), more recent studies instead indicate a prevalence of about 1% (APA, 2013; Baird, 2006; Christensen et al., 2016; Idring et al., 2015; Saemundsen et al., 2013). Similarly, the Center for Disease Control and Prevention also reported that 1 in 59 eight-year-olds in the United States has ASD (Baio et al., 2018). Regarding gender, autism is more common in boys than in girls, with a ratio of about 4: 1 (Eisenberg and Kanner, 1956; Fombonne, 2003). Recent studies suggest that girls with ASD tend to exhibit fewer repetitive and stereotyped behaviors (Szatmari et al., 2012; Tillmann et al. 2018), use more strategies to compensate for their limitations in social situations (Hull et al., 2020; Lai et al. 2017) and are diagnosed at a later mean age than boys (Petrou et al., 2018; Russell et al., 2011). Furthermore, not all people with autism show intellectual disabilities. Indeed, the presence of this comorbidity has decreased in recent years with the change in diagnostic criteria (Dunn et al., 2019).

1.1 Autism features

There is often nothing about the appearance of people with ASD that sets them apart from other people, but they can communicate, interact, behave, and learn in ways that are different from most other people. The learning, thinking, and problem-solving skills of people with ASD can range from gifted to severely challenged. Some people with ASD need a lot of help in their daily life, others need less. Some children start showing signs as early as a few months. Others appear to have normal development for the first few months or years of their life and then begin to show symptoms.

Although individuals with ASD are very different from each other, the disorder is characterized by fundamental characteristics in two areas: social communication and limited and repetitive sensory-motor behaviors (Khan et al., 2012). ASD is the result of early impaired brain development and neural reorganization (Bauman and Kemper, 2005) (O'Reilly et al., 2017). However, as there are no reliable biomarkers, diagnosis must be made on the basis of behavior. The American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders (DSM-5) criteria published in 2013 and showed in Table 1, were intended to make the diagnosis of ASD easier. To be diagnosed with ASD, a person must show evidence of difficulty, past or present, in each of the three social communication subdomains, and must have or have had difficulty with two of four different limited and repetitive sensory-motor behaviors.

As previously reported, the symptoms of behavioral matrix autism refer to two dimensions. The deficits found in the area of communication and social interaction range from the absence of emotional reciprocity, to the deterioration in the use of non-verbal behaviors, to the difficulty in developing or maintaining friendships, and the absence of sharing experiences. Difficulties in socio-emotional reciprocity range from an anomalous social approach to the failure of conversation reciprocity; from a reduced expression of interest, emotions or affection, to failure to initiate, terminate or respond to social interaction. Verbal and non-verbal communication are poorly integrated, eye contact or body language are abnormal, there is difficulty in understanding and using gestures and there may be a lack of facial expression and non-verbal communication.

Table 1. Diagnostic criteria for ASD in DSM-V

A. Persistent deficits in social communication and social interaction across multiple contexts, as manifested by the following, currently or by history (examples are illustrative, not exhaustive; see text): **1.** Deficits in social-emotional reciprocity, ranging, for example, from abnormal social approach and failure of normal back-and-forth conversation; to reduced sharing of interests, emotions, or affect; to failure to initiate or respond to social interactions. **2.** Deficits in nonverbal communicative behaviors used for social interaction, ranging, for example, from poorly integrated verbal and nonverbal communication; to abnormalities in eye contact and body language or deficits in understanding and use of gestures; to a total lack of facial expressions and nonverbal communication. **3.** Deficits in developing, maintaining, and understand relationships, ranging, for example, from difficulties adjusting behavior to suit various social contexts; to difficulties in sharing imaginative play or in making friends; to absence of interest in peers.
B. Restricted, repetitive patterns of behavior, interests, or activities, as manifested by at least two of the following, currently or by history (examples are illustrative, not exhaustive; see text): **1.** Stereotyped or repetitive motor movements, use of objects, or speech (*e.g.*, simple motor stereotypes, lining up toys or flipping objects, echolalia, idiosyncratic phrases). **2.** Insistence on sameness, inflexible adherence to routines, or ritualized patterns of verbal or nonverbal behavior (*e.g.*, extreme distress at small changes, difficulties with transitions, rigid thinking patterns, greeting rituals, need to take same route or eat same food every day). **3.** Highly restricted, fixated interests that are abnormal in intensity or focus (*e.g.*, strong attachment to or preoccupation with unusual objects, excessively circumscribed or perseverative interests). **4.** Hyper- or hyporreactivity to sensory input or unusual interest in sensory aspects of the environment (*e.g.*, apparent indifference to pain/temperature, adverse response to specific sounds or textures, excessive smelling or touching of objects, visual fascination with lights or movement).
C. Symptoms must be present in the early developmental period (but may not become fully manifest until social demands exceed limited capacities or may be masked by learned strategies in later life).
D. Symptoms cause clinically significant impairment in social, occupational, or other important areas of current functioning.
E. These disturbances are not better explained by intellectual disability (intellectual developmental disorder) or global developmental delay. Intellectual disability and autism spectrum disorder frequently co-occur; to make comorbid diagnoses of autism spectrum disorder and intellectual disability, social communication should be below that expected for general developmental level.

The second dimension, that of imitated and repetitive sensory-motor behavior, refers to the presence of stereotyped or repetitive movements (for example use of the same objects, echolalia, etc.), to the inflexible adherence to non-functional routines, to restricted and fixed interests hyper or hypo-reactivity to sensory stimuli or unusual interests in sensory aspects of the environment. The repetitive and restrictive behaviors observed in ASD may be the most isolating symptoms of the disease. Often children with autism crave a strict routine and are extremely upset if deviations from it occur. Each dimension is then specified in relation to gravity.

It is a condition from which one cannot heal, but on which to intervene. Early identification of autism represents an important challenge as it opens up possibilities for taking charge at an age where some developmental processes can still be modified.

1.2 Autism causes

Neuropsychological research attempts to explain the peculiar functioning of the "autistic" mind through the study of the cognitive, social and emotional skills of subjects with autism, while other lines of study analyze the functioning of the brain in search of the possible neurobiological basis of the syndrome, adopting various investigation techniques such as neuro-imaging techniques, neuro-functional and neuro-transmitting investigation to bring behavioral symptoms back to a neurophysiological basis. Many pathogenetic theories are proposed that seem to be confirmed in some subgroups of subjects with an autism spectrum disorder: abnormal development of certain brain structures and levels of connection between different areas, neurotransmitter dysfunctions in the central nervous system, immunological abnormalities, autoimmune processes, metabolic disorders.

On a morphological / histological level, some autopsy studies conducted on the brains of autistic subjects compared with normal subjects have shown that some brain structures, such as the cerebellum, the entorhinal cortex, the hippocampus and the amygdala, have increased nerve cell density and reduced size of the same (Amaral et al, 2008). Some recent studies conducted through neuroimaging techniques seem to indicate anomalies in the connections between neurons in specific brain areas such as the auditory cortex and the mirror neuron system (Rizzolatti and Fabbri-Destro, 2010), as well as variations in the volume and symmetry of the two cerebral hemispheres (Harms et al., 2010). These evidences suggest that very early defects (first trimester of pregnancy) in the organization of the brain are partly responsible for the behavioral and neurological alterations of autistic syndromes. At the cellular level, the most accredited hypotheses concern anomalies in the functioning of synapses, especially inhibitory ones, mainly mediated by γ-aminobutyric acid (GABA), as well as defects in the synthesis and release of serotonin (5HT), a neurotransmitter involved in modulation of social behaviors. Many studies also highlight alterations of the mechanisms involved in the modifications that occur inside the cells of the brain in response to the nerve stimulus, mechanisms related to intracellular calcium (Krey and Dolmetsch, 2007).

Finally, systemic anomalies have also been reported that could help explain the complex etiopathogenesis of autism. Autopsy studies and also the analysis of cerebrospinal fluid in patients have highlighted changes in the immune system and an increase in various molecules involved in inflammation processes at the level of the central nervous system (Vargas, 2005). Recently, theories have been suggested regarding the presence of maternal antibodies that would cross the placental barrier contributing to the immune and neurological abnormalities observed in autistic children (Dalton et al., 2003). According to the DSM-5 criteria listed, two of the key characteristics for identifying an autism spectrum disorder (ASD) diagnosis are: (1) persistent deficits in social communication and social interaction in multiple contexts; and (2) limited and repetitive patterns of behavior, interests or activities (Lai et al., 2014). Due to the broad nature of these definitions, a diagnosis of ASD often occurs in conjunction with other conditions. For example, motor abnormalities (79%), gastrointestinal problems (up to 70%), epilepsy (up to 30%), intellectual disability (45%) and sleep disturbances (50-80%) (Lai et al., 2014). Speech disorders are often concomitant and have even been included in the DSM-IV criteria.

ASD is now understood as a disease of complex interaction between genetics and the environment (Figure 1.1), with estimates of heritability ranging from 40 to 80% (Chaste and Leboyer, 2012).

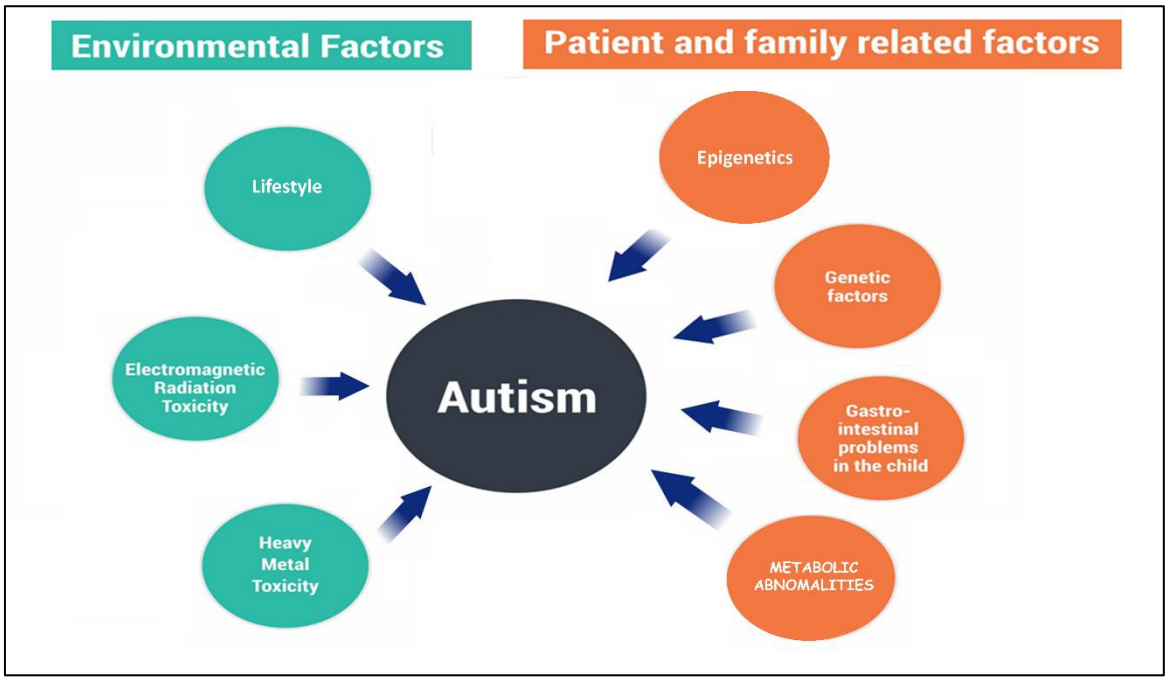


Figure 1.1: *Possible Causes of Autism Spectrum Disorder interaction between genetics and the environment.*

Extensive genetic studies have revealed hundreds of genes linked to autism. Epidemiological investigations have begun to clarify which environmental factors might contribute to risk, but much remains to be understood about how they interact with genetic predisposition to contribute to the etiology of ASD.

1.2.1 Genetic causes

The greatest advance in understanding the pathophysiology of autism has been the discovery of a significant genetic contribution to the etiology of ASD. Two main areas of evidence support a genetic etiology in ASD: comparison of monozygotic (MZ) twins and dizigotic (DZ) twins, family studies comparing the rate of autism in first-degree relatives of affected probands versus population of rare genetic syndromes diagnosed with comorbid autism. The hereditary basis of autism is mainly supported by the observation that MZ twins (who completely share the genetic makeup) are more likely to be both affected by autism than DZ twins (born from the same birth, but resulting from the fertilization of two eggs from two different spermatozoa) (Folstein and Rutter, 1977) (Bailey et al., 1995).

First-degree relatives of ASD probands have increased behavioral or cognitive characteristics related to autism, such as social or language dysfunction, albeit in minor forms, compared to the prevalence in the population (Losh et al., 2009).

Collectively, these examples suggest that the different characteristics of autism represent a quantitative continuum of functions that could be inherited. This is consistent with the awareness that specific genetic factors contribute to the development and function of specific brain structures and with the theory that distinct brain circuits may underlie different components of autism (Geschwind, 2008).

From 2003 (Volkmar et al., 2003) to the present, great strides have been made in understanding the genetic causes underlying autism. Finding significant common gene variants behind autism spectrum disorder is very difficult (Cook et al., 2008) (Veenstra-Vanderweele et al., 2004) but, after the larger Genome-Wide Association (GWAs) study, a common variant in an intergenic region between cadherins 9 and 10 that has high statistical significance was identified (Wang et al., 2009). This discovery is important as cadherins are crucial for neuronal connectivity and therefore represent a possible biological mechanism to explain the "underconnectivity theory" of autism in which the appearance of the disorder is attributed to a lower connectivity of the anatomical and functional systems between the frontal and the cortical portion. Another breakthrough in understanding the genetics of autism spectrum disorder was the discovery that variations in gene copy

numbers can also be considered a risk factor (Sebat et al., 2007). Copy number variations can be *de novo* or inherited (Cook et al., 2008) and nearly all of these are deletions, resulting in the loss of many fragments containing several genes. *De novo* copy number variations appear to be strongly associated with intellectual impairment and dysmorphology (Sebat et al., 2007). In most cases, these appear to be unique events even if we do not know all the implications, as their relationship to the phenotype cannot be directly established and the affected siblings do not always share the same specific variations (Cook et al., 2008). Furthermore, knowing whether a given *de novo* variation can be considered abnormal is difficult, as the population distribution of specific copy number variations is still unknown (Marshall et al., 2008).

The analysis of the whole genome and the cytogenetic studies carried out on families with at least two members affected by this condition have allowed to identify a certain number of genes most frequently associated with autism, located on different chromosomes such as 15, 16 , 22, and the X chromosome (Miles, 2011).

Some genetically inherited pathological conditions, such as fragile X syndrome and tuberous sclerosis, often occur in conjunction with autism. For example, malfunctions of the genes underlying fragile X (FMR1) and Rett syndrome (MECP2), imply synaptic dysfunction as a pathogenesis. Further evidence for synaptic dysfunction as a unifying cause comes from the discovery of rare mutations, in neural cells, in genes encoding synaptic adhesion molecules such as X-linked neuroligin 4 (NLGN4X) and neuroligin 3 (NLGN3) (Tabuchi et al., 2007). Evidence of genetic findings regarding implications for synaptic mismatch is particularly important as they coincide with results from neuroimaging analyzes, which also suggest that brain structural and functional connectivity is aberrant in patients with autism spectrum (Minshew et al., 2007). Therefore, both genetic and neurobiological evidence indicate, as a good causal model of this disorder, an incorrect maturation and development of synaptic connectivity.

However, the knowledge deriving from these types of studies is rapidly evolving and the involvement of genes located on different chromosomes cannot be excluded. In general, to date, genetic analysis has failed to prove evidence of involvement of a single gene associated with autism and there is consensus in indicating a complex genetic origin, characterized by the involvement of many genes. Some of these genes are involved in the

production of important molecules with a role in the development and maintenance of nerve networks. These are proteins that participate in the functioning of the nerve synapse, factors that regulate the expression of genes, neurotransmitters and their receptors, and genes involved in brain development (De Rubeis et al., 2014).

None of the genes identified so far in particular groups of patients and / or their families is in itself capable of explaining all the symptoms of this complex neuropsychiatric pathology. Researchers need to highlight how genes that influence synapse maturation can explain specific behaviors and altered functions. Synaptic maturation problems are not ubiquitous in the brain, and genes that influence brain function can also be expressed regionally, affecting only certain parts. An important implication of this model is that preventive action could be taken during the first months of life. With Drosophila models for fragile X syndrome and mouse models for Rett syndrome, researchers have already shown that the phenotype can be altered through the administration of metabotropic glutamate antagonists (in the fragile X model) (McBride et al., 2005), or the reintegration of the *MeCP2* gene after birth (in Rett syndrome) (Guy et al., 2007).

For most children, the onset of ASD symptoms is gradual; however, about 30% have an obvious "regressive" onset. These children start talking and then, gradually or precipitously, lose their tongues and drift away. Although it has been debated whether these children are well and then harmed by some exogenous exposure, the best evidence, including retrospective analysis of first birthday videotapes and neuropathological studies, suggests that their regressive course is genetically determined (Stefanatos, 2008) (Lord et al., 2004) (Volkmar et al., 2005).

However, it is possible that in some cases these combinations of "defective" genes give rise to the disorder only following environmental triggering factors, such as exposure to infectious agents during prenatal life, maternal-fetal immunological status, exposure to drugs or toxic agents including through diet during pregnancy. The role of genes is therefore increasingly interpreted as an important comorbid factor that interacts with other factors, generically called environmental, in determining the onset of the syndrome.

1.2.2 Epigenetic causes

The term epigenetics refers to heritable reversible changes in the regulation of gene expression by modulation rather than by changes in the nucleotide sequence of a gene (Liu et al., 2011). This can result in a variety of phenotypes, with both physiological and pathological consequences. There is growing evidence that aberrant epigenetic mechanisms play a role in autism spectrum disorders (ASD) (Schanen 2006). Abnormal methylation patterns such as epigenetic defects have been implicated in idiopathic ASDs (Jiang et al., 2004), as well as in ASD-associated syndromes (Rett syndrome, Angelman syndrome, Prader-Willi syndrome and Fragile X syndrome) (Schanen, 2006). Two of the main processes involved in epigenetic regulation are histone modification and DNA methylation / demethylation. Methylation is the process of transferring a single carbon atom and three hydrogen atoms, collectively defined as a methyl group, from one substance to another. Methyl group transfer regulates countless biochemical processes, including cell division, gene expression, DNA and RNA synthesis, nervous system development, immune cell differentiation, neurotransmitter synthesis, histamine clearance, and detoxification. Optimal methylation and its opposite process, demethylation, are essential for maintaining our physical, mental and emotional health; consequently, methylation deficiencies have many negative effects on the body. Conditions associated with impaired methylation include fatigue, difficulty in losing weight, depression, anxiety, hormonal imbalances, poor detoxification skills, infertility and an increased risk of developing cancer. Growing research in the field suggests that there is a connection between impaired methylation and autism spectrum disorder (ASD) (Piyathilake et al., 2011; Wong et al., 2014). The molecular changes that are involved in the development of ASD can be regulated by mechanisms that are, in part, modulated by environmental signals. In this scenario, epigenetic mechanisms, such as DNA methylation and histone modifications, could be the bridge between environmental influences and the phenotype manifested through the regulation of gene expression (Rosenberget al., 2009; Hallmayer et al., 2011). Brain development is a complex and plastic process that requires the regulated expression of specific gene sets coordinated in space and time. This task is fulfilled by transcription factors and epigenetic machinery. It is emerging that epigenetic modifications, both in DNA

and in histone proteins, underlie the fundamental mechanisms by which neurons adapt their transcriptional response to evolutionary and environmental stimuli. Failure to regulate these mechanisms, caused either by genetic and / or environmental factors, can lead to cognitive deficits and other characteristics typical of neurodevelopmental disorders. Of particular interest, studies on genome sequencing in human psychiatric and neurodevelopmental disorders have revealed mutations in many chromatin regulators. For example, the mutation in the methyl binding protein CpG 2 (MECP2), a protein product that binds methylated CpGs, causes Rett Syndrome, a disorder that phenotypically overlaps with ASD (Boris et al., 2004). Therefore, there is a direct link between the machinery linked to DNA methylation and typical autistic behavior. Although recent genomic analyses provide evidence of epigenetic alterations in blood, ectodermal cells, and postmortem ASD brain, in the field, there is a lack of systematic investigation showing a direct implication of DNA methylation and failure to regulate development-related gene expression of the ASD. In recent years, some studies have revealed that polymorphisms of genes involved in the folate/homocysteine pathway are risk factors for autistic children (Boris et al., 2004) (Mohammad et al., 2009). There is a great deal of evidence to suggest that DNA methylation defects are associated with ASDs and that the role of the *MTHFR* (methylenetetrahydrofolate reductase) gene in folate metabolism may contribute to the epigenetic mechanisms that modify complex gene expression, thus causing the autism.

Variants in the *MTHFR* gene and the presence of folate receptor autoantibodies are two factors that mediate the relationship between methylation and autism. The *MTHFR* gene regulates the production of methylenetetrahydrofolate reductase, the enzyme that converts 5-10 methylenetetrahydrofolate to 5-methyltetrahydrofolate (5-MTHF), the active form of folate. 5-MTHF is a compound that participates in methylation and an insufficiency of 5-MTHF can alter methylation by negatively affecting brain function (Hallmayer et al., 2011). Genetic variants of MTHFR, such as the C677T variant, reduce the activity of the MTHFR enzyme and lower the level of 5-MTHF in the body. The frequency of the T allele of the C677T variant, which reduces MTHFR activity, is higher in children with ASD than in non-autistic children; this finding suggests that 5-MTHF insufficiency and impaired methylation play a major role in autism. Folate receptor autoantibodies (FRAAs) also affect methylation and ASD.

Recent research has suggested that several physiological abnormalities such as mitochondrial metabolism, redox dysfunction and immune abnormalities, alone or in combination, are related to the aberrant biological processes that underlie ASD (Frye and Rossignol, 2016). Folate is a water-soluble B vitamin that is essential for numerous metabolic reactions and for normal neurological development (Greenblatt et al., 1994) (Black, 2008). It is related to many of the physiological systems found abnormal in ASD (Frye and James, 2014) and has been shown to have a protective role against the development of autism when supplemented in adequate amounts during or before pregnancy (Schmidt et al., 2012; Suren et al., 2013). In some individuals with ASD, the primary mechanism that carries folate across the blood-brain barrier may be compromised. The folate alpha receptor (FRα), along with energy-dependent endocytosis, carries folate attached to the FRα from the apical side to the basolateral side of the choroid plexus endothelium against a concentration gradient. Active folate transport is necessary because the folate concentration in the central nervous system is several times higher than the folate concentration in the blood (Frye et al., 2013). Folate receptor autoantibodies (FRAA) bind to FRα in the brain, thus interfering with folate transport across the blood–brain barrier. Blocked folate receptors cause brain folate deficiency, which impairs brain methylation and function. Autistic children have a high prevalence of FRAA compared to non-autistic children. In children with folate receptor autoantibodies, folate must be delivered to the brain via a pathway that bypasses the blocked receptor. Folinic acid, an unmethylated form of folate, easily crosses the blood-brain barrier and has been shown to improve verbal communication in children with ASD (Frye et al., 2018). Research into the relationship between MTHFR variants, folate receptor autoantibodies, and ASD suggests that administering 5-MTHF and folinic acid may help correct methylation deficits and relieve symptoms in children on the ASD spectrum.

Methylation is catalyzed by several enzymes that require specific dietary micronutrients, including folate, riboflavin, B6, B12, choline, betaine, zinc and magnesium. A deficiency in these nutrients alters methylation (Anderson et al., 2012). Additionally, research indicates that a Westernized diet high in refined and processed foods alters brain methylation and cognitive function. These findings suggest that there is an important link between the consumption of an elaborate, nutrient-rich diet and autism spectrum disorder (Yokoyama et al., 2016).

1.2.3 Environmental factors

Concordance of MZ twins has been shown to reach 70% -90% for autistic behavioral traits only, and ASD is generally assumed to be due to hereditary genetic defects (Geschwind, 2009). However, this agreement could be due to exposure to environmental agents that are neurotoxic to the developing twins' brains (Chamak, 2010). Of note, a significant percentage of MZ twins are discordant (Hallmayer et al., 2011). In DZ twins, it has not been shown that the concordance was superior to that of their isolated siblings (Chamak, 2010; Hallmayer et al., 2011) and there is strong evidence that environmental factors play a role in the development of autism as much as the genetic factor (Hallmayer et al., 2011). For over a century, autism has been believed to be an inheritable genetic disease (Chamak, 2010; Hallmayer et al., 2011). Recently, Hallmayer et al. (2011) conducted one of the largest twin couple studies. They completed studies in 192 twin couples and reported that a high degree of risk for ASD in MZ was due to environmental factors and a lower risk was due to heritability or genetics.

Prenatal exposure to harmful chemicals or the occurrence of an infection during the primary gestation period of a maturing fetus can adversely affect the development of the immune system (Landrigan, 2010). Presence and exposure to harmful chemicals or infections can increase the incidence of autoimmune deficiencies in young children. Therefore, research has linked autoimmune deficiencies as one of the many trademarks of the autism spectrum disorder (Figure 1.2). Furthermore, postnatal inflammation that occurs during the primary stages of fetal development can cause irreversible effects in the cerebral and peripheral regions of the central nervous system (Hertz-Picciotto et al., 2008).

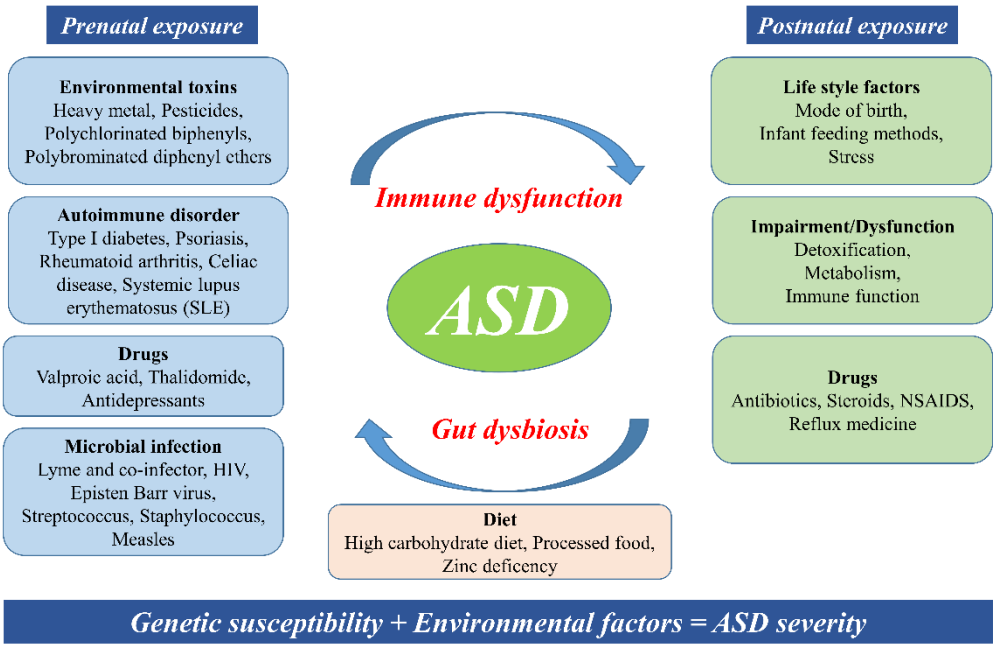


Figure 1.2: *Putative autism spectrum disorder (ASD)-related and environmental factors contributing to ASD.*

Some of the environmental factors that epigenetically influence the development of autism include diet (Kawicka et al, 2013), dysbiosis (Hughes et al, 2018), maternal infection (Zhang et al, 2010), heavy metals (Gamakaranage, 2016) and environmental toxins (Rossignol et al, 2014). Heavy metal contamination is omnipresent and children are generally more susceptible than adults; exposure to heavy metals during early development is often difficult to associate with a diagnosis of autism. Regardless, several studies have evaluated associations between autism and biomarkers after exposure to heavy metals. The evidence that heavy metal exposure leads to an autistic phenotype is increasingly compelling. Metals reactive to sulfhydryl groups, including arsenic, cadmium, lead, and mercury, are the most commonly associated metals in autism. Kern et al. (2011) evaluated hair samples from autistic children and found that levels of sulfhydryl reactive metals were lower in autistic children than in control children, suggesting that subgroups of autistic children may be more susceptible to certain metals because they are poorly excreted or detoxified, which could mean that in these subjects this substance remains accumulated in the body and is not easily excreted. Other metals can also increase the risk. Adams et al. (2013) reported that the severity of autistic symptoms can be affected by high body

concentrations of aluminum, zinc, lead and mercury as measured by urinary excretion. Further research on heavy metal exposure and autism risk is aimed at determining the most appropriate biomarkers for risk assessment (Adams et al. 2013). Several studies, carried out both on mouse models and on human nerve cell cultures, would in fact show the serious damage to the brain due to exposure to heavy metals and their connection with autism spectrum disorders in children, as well as the damage caused to neurons (Dórea et al. 2011). Bernard et al. (2001) have linked the development of autism with intoxication caused by heavy metals. All aspects of both disorders were examined and the parallelism between the two was found to be very evident (Bernard et al., 2001). First, in both cases, the subject abilities are affected, such as movement disorders, lack of coordination, difficulty in remaining seated correctly, deficits in understanding and processing speech, mental confusion and lack of concentration. But secondly, the relationship with others is also affected through the development of extreme shyness, indifference, refusal of physical contact, desire to be alone, irritability and aggression. The organ that accumulates the most heavy metals of all is the brain. Neurons also tend to be inefficient in the elimination mechanisms of these metals and this is why they are hardly expelled from the brain. Heavy metals bind to sulfhydryl molecules, preventing various cell functions, including the oxidation of sulfur compounds and the absorption of sulphates in the intestine and kidneys. Autistic children show difficulties in these processes, as well as having low levels of sulphates in the blood (Bernard et al. 2001).

The abundance of environmental toxins we are exposed to on a daily basis has a significant impact on methylation and the risk of ASD. Uterine heavy metal exposure alters gene methylation and is associated with an increased risk of autism (Kern et al., 2011). It has been shown that exposure to bisphenol-A (BPA), an extremely common environmental toxin found in plastic bottles and food containers, in the lining of canned foods and on the thermal paper used for receipts, also alters DNA methylation and is associated with an increased risk of autism spectrum disorders (Wasserman et al., 2006). An increasing number of chemicals are now implicated in the cause of neurodevelopmental disabilities, including: lead (Rosner et al., 2005; Landrigan et al, 1975); methylmercury (Harada et al, 1995); polychlorinated biphenyls (PCBs) (Jacobson et al., 1996); arsenic (Wasserman et al., 2004); manganese (Wasserman et al., 2006); organophosphate insecticides (Berkowitz et al., 2004); ethyl alcohol (Streissguth et al., 1994).

These chemicals currently identified as neurotoxic to human development could be just the tip of the potentially much larger iceberg (Landrigan, 2010). Children today are at risk of exposure to synthetic chemicals found in a wide range of consumer goods, cosmetics, drugs, fuels and building materials. In addition, they are common at hazardous waste sites and are regularly released into the air, food and drinking water. Measurable amounts of several hundred HPV chemicals are found in the blood and urine of nearly all Americans for example, as well as in human breast milk and umbilical cord blood of newborns (Reiner et al., 2007).

Interestingly, one of the most revealing aspects of a potential role for environmental factors in ASD may be the evidence of discordant MZ twins (Hallmayer et al., 2011). Since MZ twins are expected to be genetically identical, each of them should be equally vulnerable to ASD. Current evidence suggests that the autism spectrum disorder phenotype is driven by a complex interplay of genetic and environmental factors that influence brain maturation, synaptic function, and cortical networks.

1.2.4 Microbiome

The human gastrointestinal tract (GUT) is colonized by 100-400 trillion microorganisms living in a close symbiotic relationship (Sender et al., 2016). Most bacteria adhere tightly to the mucus that forms the external and internal physical barrier of the intestine over 7 m in length. Many of these organisms are transmitted in the first years of life to infants, mainly through breast milk, which contains a high number of *Bifidobacteria* and *Lactobacillus* (Palmer et al., 2012). On the other hand, in adults, most gut bacteria belong to the genera Bacteroides, Parabacteroides (Bacteroidetes) and Clostridium (Firmicutes) (Eckburg et al., 2005; Lepage et al., 2013). Aerobic pathogenic species cannot invade the intestine, as it is an anaerobic environment; however, anaerobic pathogenic species can colonize it causing disease. Each site in the gastrointestinal tract has a unique microbiota; the density of bacteria increases in the jejunum / ileus and large intestine compared to the stomach and duodenum. The highest cell density is present in the colon, approximately 10^{12} colonies per unit / mL (99%). Host and microbial communities have developed strategies that promote a balance for their mutual benefit. Dysbiosis refers to an imbalance in the abundance of microbial species relative to others, which is linked to a lower intestinal barrier function and activation of inflammatory cells (Backhed et al., 2012). Microbial diversity is likely to be at the origin and chronicity of many diseases (Clemente et al., 2013; Brown et al., 2013; Chang and Lin, 2016; Thaiss et al., 2016).

Microorganisms perform their functions primarily through enzymatic pathways to digest complex dietary carbohydrates and proteins. Thousands of microbiota-derived metabolites have been identified as components of the human metabolome (Overbeek et al., 2005). Plant foods particularly rich in dietary fiber and polyphenols are essential for maintaining a stable and fermentative intestinal microbiota. These products are processed by intestinal microbiota enzymes such as glycosidic hydrolases and polysaccharide lyases. Under anaerobic conditions, species belonging to the *Bacteroides* family and to the *Clostridiaceae* and *Lactobacillaceae* families produce short-chain fatty acids (SCFA). SCFAs play a key role in controlling proliferation, differentiation and maintaining mucosal integrity. Impaired production of SCFA, tryptophan metabolites, GABA, noradrenaline, dopamine, acetylcholine and 5-HT (5-hydroxytryptamine or serotonin), all related to microbial

metabolism, is involved in gastrointestinal abnormalities, metabolic diseases and neuropsychiatric disorders (Round and Mazmanian, 2009; Tremaroli and Bäckhed, 2012; Gilbert et al., 2016). Many of these molecules are major neurotransmitters. Specifically, serotonin has been linked to its central roles in appetite, sexuality, substance addiction, emotions and stress response. There is evidence to believe that most brain serotonin comes from the gut microbiome or has its synthesis modulated by the brain-gut axis (O'Mahony et al., 2015). In addition, the microbiota produces large amounts of epigenetically active metabolites, such as folate and vitamin A.

Surprisingly, the genomic and biochemical complexity of the microbiota exceeds that of the brain. Studies on the brain-gut-microbiota axis have been described as a paradigm shift in neuroscience (Mayer et al., 2014). Growing evidence points to a diversity in the gut microbiota that is critical, not only for gut health but also for normal physiological functioning in other organs, particularly the brain. An altered intestinal microbiota in the form of dysbiosis in both the newborn and the elderly, can have a profound impact on brain function. Such dysbiosis could arise for several reasons, such as the manner of delivery, diet and exposure to antibiotics and other drugs. The essential metabolic products that come from the gut microbiome are essential for the brain, so it is not surprising that dysbiosis can have serious negative consequences for the brain from both a neurological and mental health perspective. The brain-gut-microbiota axis is a two-way communication system that allows gut microbes to communicate with the brain and the brain with the gut (Rhee et al., 2009). The mechanisms of signal transmission are complex and not fully elucidated, but include neural, endocrine, immune and metabolic pathways (El Aidy et al., 2015; Grenham et al., 2011). The intestinal microbiota also regulates the main central neurotransmitters, such as serotonin, by altering the levels of precursors; for example, *Bifidobacterium* infantis has been shown to raise plasma levels of tryptophan and thus influence the central transmission of serotonin (5HT) (Desbonnet et al., 2010). Interestingly, *Lactobacillus* and *Bifidobacterium* bacteria can produce g-aminobutyric acid (GABA); *Escherichia*, *Bacillus* and *Saccharomyces* noradrenaline; *Candida*, *Streptococcus*, *Escherichia* and *Enterococcus* serotonina; and *Lactobacillus* acetylcholine (Lyte, 2013; 2014). These microbiota-synthesized neurotransmitters can cross the mucous layer of the gut, although they are unlikely to directly affect brain function. Their impact on brain function is likely to be indirect as they are unable to cross the blood brain barrier (BBB). They act on the enteric

nervous system. The high comorbidity between stress-related psychiatric symptoms such as anxiety with gastrointestinal disorders including irritable bowel disorder (IBS) and inflammatory bowel disorder (IBD) (Camara et al., 2009; Mawdsley and Rampton, 2006) are further evidence of the importance of this axis.

1.3 Microbiome and autism spectrum disorder

Bacterial colonization of the intestine occurs at the time of birth, when a mixture of maternal vaginal microorganisms is transferred to babies born through natural birth. Indeed, babies delivered by caesarean section may receive insufficient maternal bacterial transmission (Murgas Torrazza and Neu, 2011; Walker, 2013; Mueller et al., 2015). The gut microbiome of most babies is initially similar to that of the mother, but after 1 year of age a distinct microbiome profile develops (Mackie et al., 1999; Palmer et al., 2007). There are critical windows for the establishment of the gut microbiome and subsequently for the effects on health and disease. This time requirement may have important ramifications for potential preventive and therapeutic remedial strategies.

As previously mentioned, the gut microbiota performs several functions. For example, it synthesizes several essential vitamins and cofactors, such as folate and vitamin B 12, which can influence the methylation of DNA and histone proteins (Le Galliard et al., 2008) (LeBlanc et al., 2013). Bacteria also metabolize lipids, proteins and complex carbohydrates, including those indigestible by the host (Hooper et al., 2002) (Saulnier et al., 2008) (Dai et al., 2012). Furthermore, as pointed out above, bacterial fermentation leads to the production of various short-chain fatty acids (SCFA) (Hooper et al., 2002). Bacterial-derived SCFAs can also alter the intercellular spaces between cells, resulting in intestinal permeability that allows more metabolites and bacteria to pass through the epithelial barrier, which can lead to damaging neurological effects. SCFAs are considered key elements of the microbiota-gut-brain axis and their involvement in multiple neurological disorders is known (Dalile et al., 2019). It is interesting to note, for example, that patients with propionic acidosis, a genetic disease characterized by an accumulation of propionate, leads to neurodevelopmental delay, and recent studies have reported a very high prevalence of ASD (21%) in patients with this disease (Cotrina et al., 2020). Propionate can increase oxidative stress, thereby affecting mitochondrial activity. In fact, mitochondrial dysfunction is common in many patients with ASD and is believed to play a role in its pathophysiology (Valiente-Pallejà et al., 2018). Moreover, disturbances in the gut and other microbiomes (dysbiosis) can affect host immunity and neurobehavioral responses (Cryan and Dinan,

2012; Douglas-Escobar et al., 2013; Ding and Schloss, 2014; Galland, 2014; Stilling et al., 2014; Sherman et al., 2015).

Disorders of the gastrointestinal system are a common comorbidity in patients with ASD (Buie et al., 2010; Mayer et al., 2014). Importantly, the prevalence of gastrointestinal (GI) symptoms is four times higher in children with ASD than in healthy children, and that many studies report a specific GI phenotype in ASD, characterized by increased leaky gut and immune function in the gut (McElhanon et al., 2014). Interestingly, children with ASD with functional gastrointestinal disorders exhibit a specific gut microbiota and immune signature compared to healthy children with the same GI symptoms. Furthermore, there is a correlation between ASD-specific dysbiosis (more specifically species increase from the order *Clostridiales*) and GI symptoms (abdominal inflammation and pain) (Luna et al., 2017). This suggests that these gastrointestinal symptoms are an integral part of the pathophysiology of ASD and interact with the gut microbiota and the immune system.

Several teams have found an increase in urinary p-cresol, a tyrosine-derived bacterial metabolite, in young children with ASD (Altieri et al., 2011; Gabriele et al., 2014; Kang et al., 2018; Gevi et al., 2020). These teams speculated that this increase could be due to a higher level of p-cresol-producing bacteria such as *Clostridium difficile*. Follow-up studies have shown that *Clostridial* groups are significantly elevated in children with ASD, such as *Clostridium bolteae* (Song et al., 2004). Another study from this group revealed nine *Clostridium* species present in ASD, but not in healthy children. Non-spore-forming anaerobes and microaerophilic bacteria were abundant in the stools of children with ASD, but absent in control children (Finegold et al., 2002). *Clostridium histolyticum* (*Clostrium* cluster I and II) for example, is another toxin producing species abundant in the fecal flora of children with ASD.

New technologies, such as 16S rDNA sequencing, have revealed other alterations of the gut microbiota in children with ASD. The presence of *Desulfovibrio*, another anaerobic bacillus that possesses various virulence factors and generates various metabolic by-products has been found in children with ASD (Finegold, 2011). The same children also showed high amounts of fecal *Bacteroides vulgatus*. Dybiosis was evident at the phylum level, with autistics showing greater amounts of *Bacteroidetes* in the stool than controls. The two groups also had significant differences in the phyla *Actinobacterium* and

Proteobacterium (Finegold et al., 2010). Other studies have instead ascertained that children with ASD with and without gastrointestinal disorders possessed greater quantities of *Sutterella spp.*; whereas, *Ruminococcus* pairs were elevated in the stools of children with ASD and GI symptoms compared to those without such disorders (Wang et al., 2013).

There are associations between bacterial metabolites in stool or urine and behavioral disturbances in ASD, including children receiving antibiotic or probiotic treatment. Adams et al. (2011) reported a strong positive correlation between GI symptoms and clinical severity of ASD. In urine, free amino acids glutamate and taurine were higher in children with ASD, possibly suggesting disruptions in sulfur and amino acid metabolism. Disturbances have also been identified in the patterns of bacterial metabolites dimethylamine, hippurate and phenylacetylglutamine in patients with ASD (Yap et al., 2010). The conclusions are that changes in the gut microbe are associated with alterations in fecal SCFA concentrations and urinary amino acid and ammonia concentrations.

Overall, these meta-analyses confirm the presence of dysbiosis in ASD. This could be due to methodological differences, but also to the fact that the different analyses come from multiple countries with different lifestyles and eating habits. Additionally, the ages of the children recruited across the different studies vary significantly, with some including 2-year-olds, an age when the gut microbiota is not fully stabilized (Stewart et al., 2018). Despite this, there appears to be a rather large increase in *Clostridium*, which is considered a purported harmful genus. It is also interesting to point out that between the different studies the control groups differed in their constitution. Taking into account both siblings of children with ASD and unrelated individuals as controls, the result is that the sibling group appears to have a different microbiotic profile than unrelated individuals and is sometimes closer to the ASD profile (Parracho et al., 2005; Wang et al., 2011; Wang et al., 2013; Tomova et al., 2015). This is not surprising considering the influence of genetics and the environment on the composition of the gut microbiota.

2. The omics sciences

The omics sciences are the set of disciplines that study the molecular mechanisms within cells (Prakash et al., 2010) (Figure 2.1).

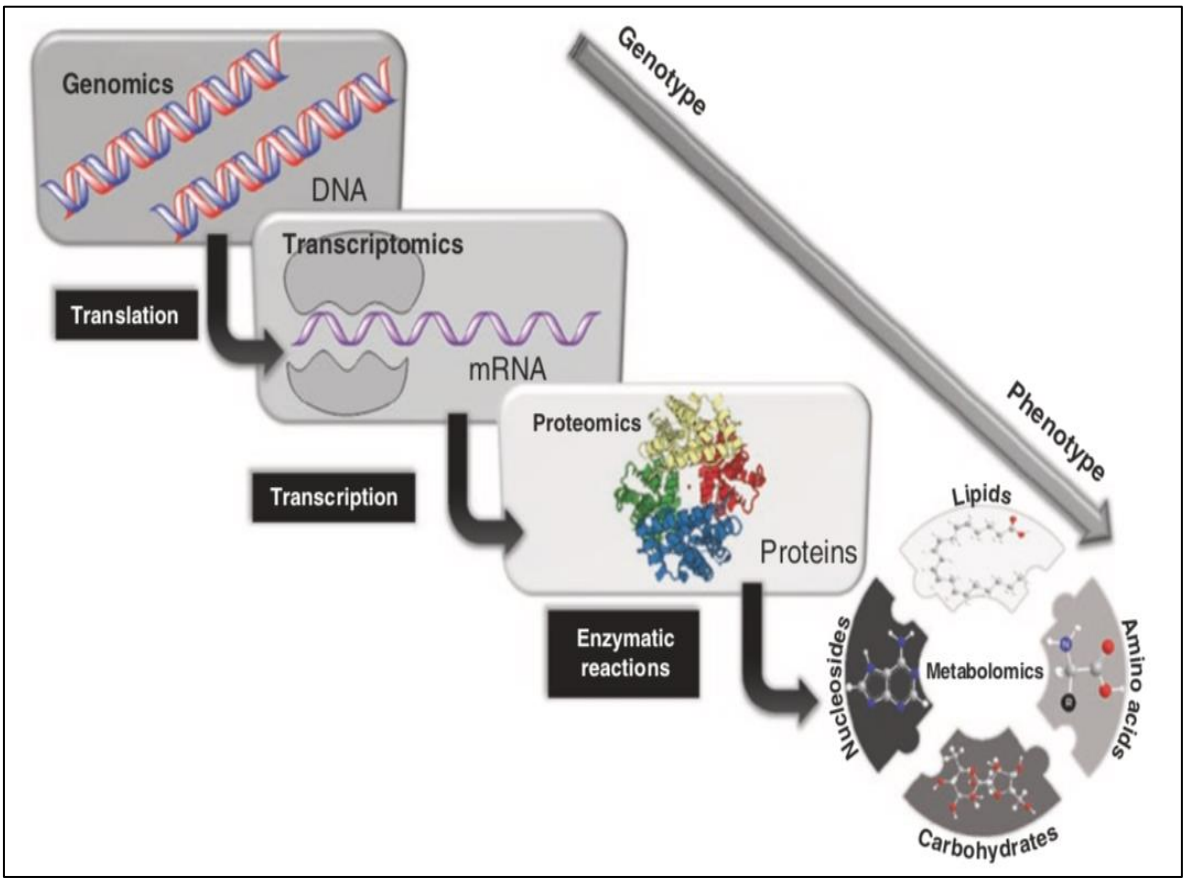


Figure 2.1: *Correlation between the main omics strategies used in the study of systems biology.*

The "omics" techniques, increasingly used in the last two decades (Boja et al., 2014), have made it possible to clarify the different aspects of cells, improving knowledge in molecular biology technologies. The suffix "omic" refers to a field of study in molecular biology including genomics, transcriptomics, proteomics, metabolomics or lipidomics which study the genome, proteome, transcriptome, metabolome, or lipidome, respectively. Specifically,

genomics is the science that studies the structure, function, evolution and mapping of genomes and aims at the characterization and quantification of genes. The transcriptome represents the set of all molecules of messenger RNA in a cell, tissue or organism. Proteomics is the science that studies proteins in relation to their biochemical properties and functional roles, and how their quantities, modifications and structures change during growth and in response to internal and external stimuli. Metabolomics is the science that studies all chemical processes involving metabolites, which are the end products of cellular processes, in a biological cell, tissue, organ or organism. Finally, lipidomics studies on a large scale the pathways and networks of cellular lipids in biological systems. However, while the observed changes in the transcriptome or proteome do not always correspond to phenotypic alterations (Gygi et al., 1999;Sumner et al., 2003), the measurement of metabolites or lipids synthesized by a biological system constitutes an important complement for evaluating genetic function (Villa boas et al., 2005).

Until a few years ago, traditional biology has focused on the identification of individual cellular components and on the study of their functions separately, with an approach that is defined as "reductionist". In recent years, however, the awareness has emerged that a biological system is the sum of several parts and its functioning cannot be reflected by the function of a single component. Therefore, unlike reductionism, systems biology promotes a "holistic" approach: a biological system is seen as a complex network of dynamic interactions between its components, such as genes, mRNAs, proteins, metabolites and lipids, studying the whole "omic cascade" (Prakash et al., 2010; Bremer, 1983). In the related fields of proteomics, metabolomics and lipidomics, liquid chromatography analysis combined with mass spectrometry (LC-MS) is becoming increasingly important as it improves the efficiency, sensitivity and reliability of targeted and non-targeted analyses. Mass spectrometry (MS) represents an universal and sensitive tool that can be used to characterize and quantify a large number of compounds in a biological sample (Kaddurah-Daouk et al., 2008). MS measures the mass-charge ratios (m/z) of ions to determine their molecular weight (MW) thus obtaining the molecular identification and quantification of polar, less polar and neutral compounds, even when they are present at relatively low concentrations and in a complex matrix (Villa boas et al., 2005). This process involves several steps:

- The sample is brought to the mass spectrometer via a chromatographic device (*e.g.* LC column)

- Ionization and vaporization of molecules into gaseous ions by the ionization source;

- Ion separation based on their m/z values by magnetic or electric fields through a component, *i.e.* the mass analyzer;

- The detection of the separated ions as electric charge obtaining signals proportional to the abundance of each species and a plot of ion abundance against m/z represents a mass spectrum (Griffiths and Wang, 2009) where in the x axis the m/z values are reported, while the y axis indicates the total number of ions.

In many configurations, additional tandem MS (MS/MS) scans are possible. In MS/MS mode, the instrument uses the first mass analyzer to select a single ion which is subsequently directed into a collision cell, where it collides with gas molecules such as argon (*e.g.*, collision-induced dissociation, CID) which causes the ion fragmentation. As this extraordinary analytical technology can provide key information about analytes (structure, purity and composition), mass spectrometry is becoming increasingly accepted as a routine diagnostic tool in clinical laboratories (Tenori et al., 2012) in the industry and field of research for various purposes such as drug discovery, diagnostics and bioanalysis (Di Girolamo et al., 2013).

2.1 Metabolomics

Metabolomics is the science that studies all metabolites (molecules <1 kDa) within a biological system (Carraro et al., 2009). The metabolome represents the whole set of metabolites that belong to a wide variety of different classes, such as amino acids, lipids, organic acids, nucleotides, etc. (Dettmer et al., 2007). The metabolome is characterized by a great variety of chemically different molecules ranging from ionic inorganic species to hydrophilic carbohydrates, volatile alcohols and ketones, amino and non-amino organic acids, hydrophobic lipids and complex natural products (Villa boas et al., 2005). Metabolomics, by studying and quantifying the metabolites present in biological fluids, offers an instant view of the system, providing useful information for understanding the processes taking place in the analyzed organism (Figure 2.2).

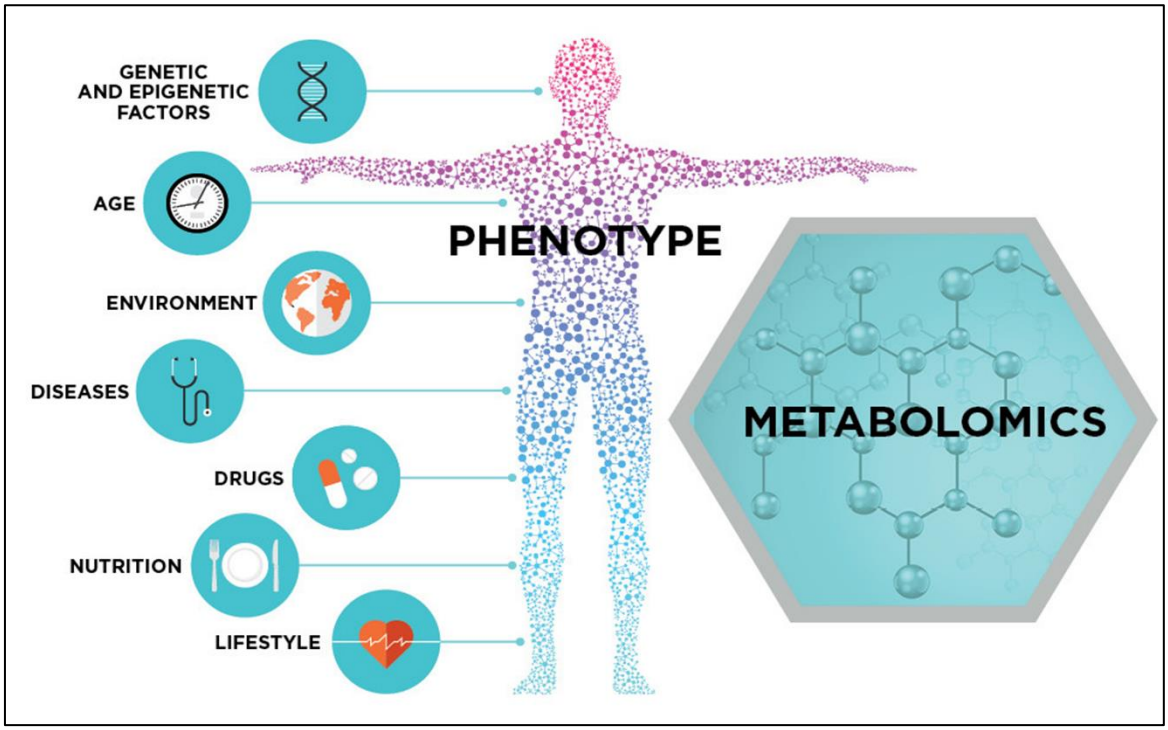


Figure 2.2: *More than 40,000 metabolites are known to be in the human body. Metabolites that appear in biological fluids provide very useful information about a patient's current health.*

Thanks to the close correlations of the metabolome with the genotype, physiology and environment, this science offers the unique opportunity to define the correlations between genotype and phenotype and the relationships between phenotype and environment (Gomase et al., 2008; Griffin et al., 2004; Eden et al., 2010).

Another feature of the metabolome is its highly dynamic nature: this allows the metabolome to be a very rapid indicator of the perturbations of a system (Bremer et al., 1983). Metabolomics allows the identification of both metabolic patterns and single metabolites, useful for understanding the etiology of a disease and for following its progression over time, especially in the context of multifactorial diseases. Furthermore, the identification of unexpected and unknown metabolites can allow to formulate new pathogenic hypotheses (Nicholson et al., 2008; Mamas et al., 2011; Van der Greef et al., 2004). For the study of metabolites different complementary methodologies are used, for each level of information. The first is a highly specific approach that focuses on the qualitative and quantitative analysis of a single analyte, such as a disease marker or the substrate of an enzymatic reaction (Dettmer et al., 2007). This is referred to as "targeted analysis". On the contrary, the “metabolite profiling” is instead an approach focused on the analysis of a group of metabolites belonging to a specific metabolic pathway or to a certain class of compounds. The analysis of the targets and the metabolic profile are generally hypothesis-driven approaches, therefore based on previous knowledge, as well as selective, as they are focused on a limited number of metabolites; they are therefore not considered a true type of metabolomic approach (Carraro et al., 2009; Dettmer et al., 2007). These approaches have the advantage of limiting the analytical and statistical load of an experiment, even if there is a risk of losing perturbations of secondarily interconnected pathways. The "metabolite fingerprinting" is instead an approach that starts from the evaluation of the largest number of metabolites possible, for the definition of metabolic patterns or "fingerprinting" associated with a specific condition. The primary purpose is not the identification of the single metabolite, but rather the definition of the metabolic characteristics capable of discriminating between the groups under examination. The fingerprinting of metabolites is a real omic approach, because it guarantees a global study of the metabolites, not guided by a priori hypotheses. This type of analysis provides a numerical representation of the data obtained which constitutes a metabolic fingerprint. Untargeted metabolomics, on the other hand, has the advantage of revealing unique and

previously unrecognized changes in metabolites and enzyme pathways, although the amount and complexity of the experimental data can be challenging (Goodacre et al., 2004; Schrimpe -Rutledge et al., 2016). Once the specific pattern of a certain condition has been identified, the structural identification of the relevant metabolites is carried out: this allows the identification of significant biomarkers in the distinction between different pathophysiological conditions. First of all, the possibility of identifying unexpected or even unknown biomarkers is left open, which can pave the way for a better understanding of not fully understood pathophysiological mechanisms and become the target of new therapies (Nicholson et al., 2002; Madsen et al., 2010).

2.2 Metabolomics and autism spectrum disorder

Due to the heterogeneity associated with ASD, enormous efforts have been made to identify underlying mechanisms or markers for this complex set of disorders with little success. Genetic variation, for example, is believed to account for approximately 50% of the risk of ASD (De Rubeis et al., 2015). While some cases are thought to be due to common polymorphisms (Klei et al., 2012; Chaste et al., 2017), specific molecular diagnoses, such as copy number variants or monogenic disorders, are nevertheless found in an estimate from 30% to 40% of children with ASD, suggesting the American College of Medical Genetics and Genomics to recommend at least chromosomal microarray and fragile X testing as a first diagnostic step (Schaefer et al., 2013). A small fraction of ASD cases also occur in patients with known inborn errors of metabolism (IEM) (Ghaziuddin and Al-Owain, 2013; Geschwind and State, 2015). The discovery of this association and the potential opportunity for therapeutic intervention have prompted the renewed search for characteristic neurometabolic biomarkers to aid in early screening and diagnosis of patients. The identification and use of a specific biomarker would therefore help differentiate these patients and would also help streamline the diagnostic process. One of the newest and most promising techniques available for identifying such biomarkers is non-targeted metabolomics.

The variety of metabolic disorders identified in patients seems to suggest that ASDs may represent a common endpoint for multiple different abnormal neuronal pathways. The choice of sample type is often crucial. Most autism metabolomics studies have used urine samples, possibly because urine sampling in children and infants is non-invasive. Most of the studies have operated on the assumption that children with ASD are largely biochemically homogeneous; however, these individuals are more likely to exhibit a myriad of biochemical phenotypes similar to the large heterogeneity observed clinically. While it would be ideal, for example, to have a single or even a small number of marker molecules to help definitively distinguish these patients from controls, the answer most likely might lie in identifying larger perturbations or path patterns.

Untargeted metabolomics appears to be a promising research tool and has the potential to make significant discoveries about the underlying characteristics of autism. Urine and plasma studies can be further optimized by more rigorous characterization of patients' diets, supplement intake, and even the microbiome. Minimizing or even eliminating these types of variables can have a significant effect on the interpretation of metabolomic analyses. There is also a great opportunity to combine metabolomic and genomic data for an integrated and comprehensive approach to both diagnosis and ongoing management.

3. A metabolomics approach to investigate urine levels of neurotransmitters and related metabolites in autistic children

3.1 Introduction

Autism spectrum disorder (ASD) is a complex syndrome characterized by communicative deficits, as well as stereotyped and repetitive patterns of behaviour (DiCicco-Bloom, et al. 2006) (Llaneza et al., 2010) (Lai et al, 2010). Autism affects more males than females, with a ratio of about 4:1 (Cohen, 2004) (Baron-Cohen et al., 2011). About 20 years ago, the incidence of autism in the US was 1 in 10,000 births, so it was considered an uncommon disorder (Hertz-Picciotto and Delwiche, 2009) (Currenti, 2010), while in 2018, the Centers for Disease Control and Prevention (CDC) estimated that the incidence of autism in the US had reached 1 in 59 births (Centers for Disease Control and Prevention, 2018).

Currently, the diagnosis of autism is generally carried out through clinical observation, using the criteria of the fifth edition of Diagnostic and Statistical Manual of Mental Disorders (DSM-5) (American Psychiatric Association, 2013). The importance of finding biochemical biomarkers in biological fluids that can diagnose autism at a younger age, without invasive methods, could help clinicians to provide earlier and more reliable diagnoses. In this regard, it is of note that besides the contribution of genetic and environmental factors in the aetiology of ASD (Kim and Leventhal, 2015) (Colvert et al., 2015), recently particular attention has been paid to the intestinal microbiome of autistic subjects, which is different to healthy children, making critical contributions to the metabolism of ASD subjects. On the other hand, the human adult gut contains an amount of bacteria equal to the weight of the human brain (Dinan et al., 2015) and it is estimated that the gut contains >100 times as many genes as in the human genome (Stilling et al., 2014), suggesting that the biochemical and genomic complexity of the microbioma overcome that of the human brain. Thus, it is not surprising that in autism spectrum disorders (ASD) the gut microbiome may play a role in regulating brain function and other metabolomics abnormalities. In our recent investigation (Gevi et al., 2016), we showed that in autistic subjects tryptophan is preferentially transformed to xanthurenic acid and

quinolinic acid instead into kynurenic acid and melatonin in agreement with Boccuto et al. (Boccuto et al., 2013). More interestingly, the gut microbiome contributes to altered tryptophan metabolism, yielding increased concentrations of indolyl 3-acetic acid and indolyl lactate, inducing an overexpression of quinolinic acid, reduction of melatonin and gut dysbiosis. Moreover, a concomitant deficiency of three B vitamins, B6, B9 and B12, was found in a significant number of autistic subjects (Belardo et al., 2019), that may cause methylation deficiency of DNA (Schafer and Buettner, 2001). This deficiency was again explained as related to intestinal dysbiosis, which is the main cause of vitamin intestinal malabsorption (Berding and Donovan, 2016) (James and Holloway, 2005). In fact, mammals are disable to synthesize many key nutrients; therefore the gut microbiota becomes fundamental for digestion, synthesizing essential dietary vitamins and cofactors, such as vitamin B, riboflavin, thiamine, and folate. It is of interest to investigate if in autistic subjects the different microbiota can affect, through the so-called "microbiome-gut-brain axis" (Barajon et al., 2009), neurotransmitters production. This could be expected, since in healthy children the microbiome plays a role in maintaining immune homeostasis, regulating the production of serotonin, dopamine, kynurenine, γ-aminobutyric acid, SCFAs, 4-cresol (Ray, 2014), and controlling the activities of the central nervous system (CNS) (Sampson and Mazmanian, 2015). In support, recent human epidemiologic studies linking alterations in the gut microbiota to ASD have been done (Son et al., 2015), suggesting that gut microorganisms could affect the central nervous system (CNS), although the mechanisms were not fully understood.

During the last decade, many investigations have been performed by using metabolomics, which are aimed at identifying the global biochemical metabolisms of autistic individuals, in an attempt to determine the alterations of specific metabolic pathways (Dieme et al., 2015) (Ming et al., 2012) (Yap et al., 2010) (Noto et al., 2014). Metabolomics' technologies offer a sensitive means to identify specific biological pathways and can be applied to investigate several human diseases, to improve their diagnosis and design better therapeutic strategies. Moreover, these approaches, in association with multivariate and univariate statistical analyses, can identify differences in small molecule abundance that may have the ability to delineate some forms of ASD, providing a specific and personalised profile in autistic subjects.

In the present study, a mass spectrometry-based method, consisting of an UHPLC coupled with a orbitrap Q-exactive, was applied for the high-throughput profiling of metabolite levels in the urine of ASD children. We chose the urine as the biological fluid for our analyses because it is easier to recruit and is a less invasive operation for the subjects. In particular, in this investigation, we used a large non-targeted metabolomics platform and different samples in order to provide greater coverage of the metabolites than previous works (Gevi et al., 2016) (Belardo et al., 2019). This allowed us to discuss the altered neurotransmitters observed in young autistic subjects. High levels of dopamine and its metabolite, homovanillic acid were found, suggesting a dopamine β-hydroxylase enzyme block, probably due to the presence of 4-cresol together with higher levels of vitamin C. Consequently, lower noradrenaline and adrenaline as well as MHPG and vanillylmandelic acid were produced, as here revealed. Moreover, less glutamate was biotransformed into γ-aminobutyric acid (GABA), due to the lower levels of pyridoxal phosphate present in autistic subjects.

3.2 Materials and Methods

3.2.1 Participants

The diagnosis of autism was formulated according to the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (American Psychiatric Association, 2013). The research was not a clinical trial and the procedure was in accordance with the Declaration of Helsinki of the World Medical Association. Forty children with idiopathic ASD and forty typically developing controls (aged 3–8 years) were recruited in Italy and northern Europe. Their demographic and clinical characteristics are summarized in Table S1. Diagnostic assessments and medical screening have been previously described (Gevi et al., 2016). Tight sex- and age-matching (±1 year) was applied to recruit typically developing children devoid of any overt ASD symptomatology among the offspring of clinical/academic personnel (Gevi et al., 2016). The mean age (±SD) of both cases and controls was 4.95 ± 0.45 and 4.35 ± 0.55 years, respectively, and the M:F ratio was 31:9 and 29:11, respectively. These analyses were considered exploratory.

Table S1. *Clinical characteristics of the autistic subjects.*

	N	Mean/Median	Range
Age in yrs (mean± S.E.M.):	N=40	4.95 ± 0.45	3-8
Median ADOS scores:	N=28		
1) Language and communication		4.90	0-10
2) Social interactions		8.80	4-13
3) Play and imagination		3.30	1-5
4) Stereotypies		3.10	0-6
5) Abnormal behaviors		0.30	0-2
Median ADI scores:	N=12		
A) Reciprocal social interactions		18.50	5-30
B) Language/Communication		13.80	9-23

C) Restricted, repetitive and stereotyped behaviors and interests			5.20	2-9
D) Behavioral abnormalities at or prior to 36 months of age			4.30	3-5
		N	**Percent**	
Gender:	*Male*	31	77.5%	
	Female	9	22.5%	
	M/F ratio	3.4:1		
Family type:	*Simplex*	36	90.0%	
	Multiplex	4	10.0%	
DSM-IV Diagnosis:	*Autistic Disorder*	40	100.0%	
	Asperger Syndrome	0	0.0%	
	PDD-NOS	0	0.0%	
I.Q. (N=40):	*>70*	12	34.8%	
	≤ 70	28	65.2%	

3.2.2 Urine collection and metabolite extraction

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

3.2.3 Ultra High-Performance Liquid Chromatography – Mass Spectrometry

Twenty microlitres of supernatants were injected into an Ultra High-Performance Liquid Chromatography (UHPLC) system (Ultimate 3000, Thermo) and run in positive and negative ion mode. A Reprosil C18 column (2.0 mm × 150 mm, 2.5 μm – Dr Maisch, Germany) was used for metabolite separation. Chromatographic separations were achieved at a column temperature of 30 °C and flow rate of 0.2 mL/min. A 0–100% linear gradient of solvent A (ddH_2O, 0.1% formic acid) to B (acetonitrile, 0.1% formic acid) was employed over 20 min, returning to 100% A in 2 min and with a 6-min post-time solvent A hold. The UHPLC system was coupled online with a mass spectrometer Q Exactive (Thermo) scanning in full MS mode (2 μscans) and in MS/MS mode at a resolution of 70,000 in the 67 to 1000 *m*/*z* range, a target of 1×10^6 ions and a maximum ion injection time (IT) of 35 ms. Source ionisation parameters were: spray voltage, 3.8 kV; capillary temperature, 300 °C; sheath gas, 40; auxiliary gas, 25; S-Lens level, 45. The identifications of the metabolites are also detected by the mass spectra provided by the MS/MS fragmentation. Calibration was performed before each analysis against positive and negative ion mode calibration mixes (Piercenet, Thermo Fisher, Rockford, IL) to ensure the sub ppm error of the intact mass.

3.2.4 Metabolomics data processing and statistical analysis

Data were normalized by urinary specific gravity, because creatinine excretion may be abnormally reduced in ASD children. Raw files were exported and converted into mzXML format through MassMatrix (Cleveland, OH), and then processed by MAVEN.52 (available at http://genomics-pubs.princeton.edu/mzroll/); spectrometry chromatograms were assessed for peak alignment, matching and the comparison of parent and fragment ions, and tentative metabolite identification (within a 2 ppm mass-deviation range between observed and expected results against the imported KEGG database). Metabolomics data of control or autism samples were analysed using XCMS Online. XCMS extracted metabolomic features with statistically significant expression changes among the two groups to produce a list of differentially expressed features based on *p*-values ($p \leq 0.05$,

≥1.5-fold change) (Smith et al., 2006). Features were putatively identified using tandem mass spectra searched against the following databases: METLIN, Human Metabolome Database (HMD). Results were graphed with GraphPad Prism 5.01 (GraphPad Software Inc.). Unpaired *t*-test was performed with the same software. Data are presented as the means ± SD.

3.2.5 Results

ASD-specific urinary metabolomic patterns were explored in 40 ASD children and 40 matched controls using untargeted metabolomics through UHPLC-mass spectrometry (Q-exactive analyser), and by using XCMS Metlin software for data interpretation. Through this new advanced technique, a more considerable number of urinary altered metabolites (about 16,704 features) were recorded in autistic children than in the previous investigations, which allowed us to collect metabolites involved in neurotransmitter production. Among these metabolites, XCMS software identified 222 statistically significant metabolites, which were up-regulated (green circles) or down-regulated (red circles) in autistic samples (Figure 3.1).

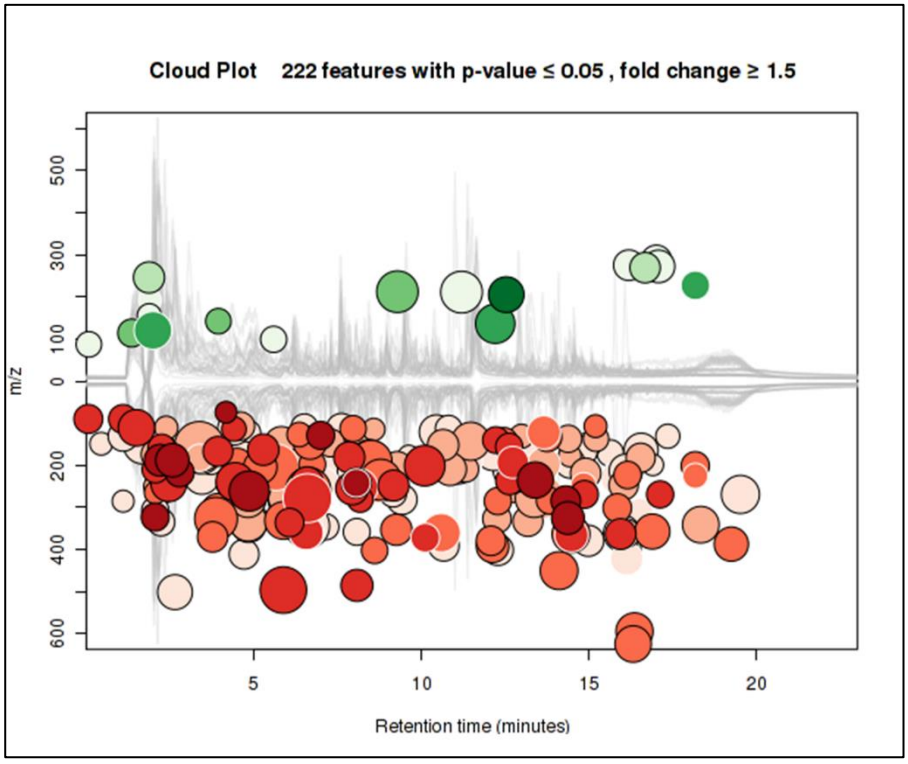
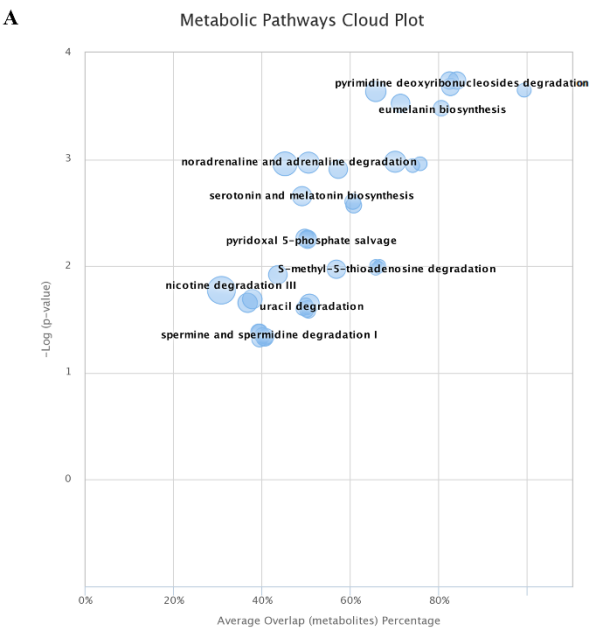


Figure 3.1: *Cloud plot of global metabolomic analyses showing up- and downregulated metabolic features comparing urine samples of autistic children with control, 222 were found to be dysregulated with a p-value ≤ 0.05 and fold change ≥1.5. The size of the circle represents fold change, colour intensity p-value, and position within the plot provides m/z and RT information.*

The size and colour of the circles correspond to the log fold change and *p*-values, respectively. Furthermore, by using the XCMS maps, the changed metabolic pathways, as a consequence of the 222 differential metabolites, were identified and represented in a Metabolic pathways Cloud Plot (Figure 3.2, Panel A). The blue circles represent the altered paths with a *p*-value < 0.05. The circles in upper side of the x-axis have greater statistical significance while on the right on the y-axis have the largest number of metabolites belonging to the same metabolic pathway. Figure 3.2, Panel B reports, in decreasing order of statistical significance, the name and the *p*-value of dysregulated pathways. Figure 3.2 shows that, beside the altered metabolic pathways such as tryptophan, purine-pyrimidine pathways and pyridoxal 5-phosphate salvage, discussed in our previous papers (Gevi et al., 2016) (Belardo et al., 2019), we now show that the noradrenaline and adrenaline degradation is significantly affected and will be further discussed in this paper.



B

Pathway name	p-value
Pyrimidine deoxyribonucleosides degradation	0,0002
Eumelanin biosynthesis	0,00035
Noradrenaline and adrenaline degradation	0,0011
Serotonin and melatonin biosynthesis	0,0023
Pyridoxal 5-phosphate salvage	0,0059
S-methyl-5-thioadenosine degradation	0,011
Nicotine degradation III	0,016
Uracile degradation	0,024
Spermine and spermidine degradation	0,046

Figure 3.2: *Using the 222 differential metabolites, XCMS maps the metabolites to metabolic pathways represented on a cloud plot (Panel A). Pathways with greater percent overlap of metabolites and statistical significance will appear in the upper right corner of the cloud plot. Nine pathways were found to have a p-value < 0.05 and their predicted dysregulation is shown in the table (Panel B).*

Figure 3.3 shows that in autistic children the higher the amount of 4-cresol in the urine and the higher the measured concentration of dopamine, suggesting that 4-cresol may play a role in the dopoamine accumulation. This allows one to exclude that the accumulation of dopamine in autistic children is not related to its greater production or to the blockage of certain enzymes, such as MAO or COMT enzymes, and other reasons.

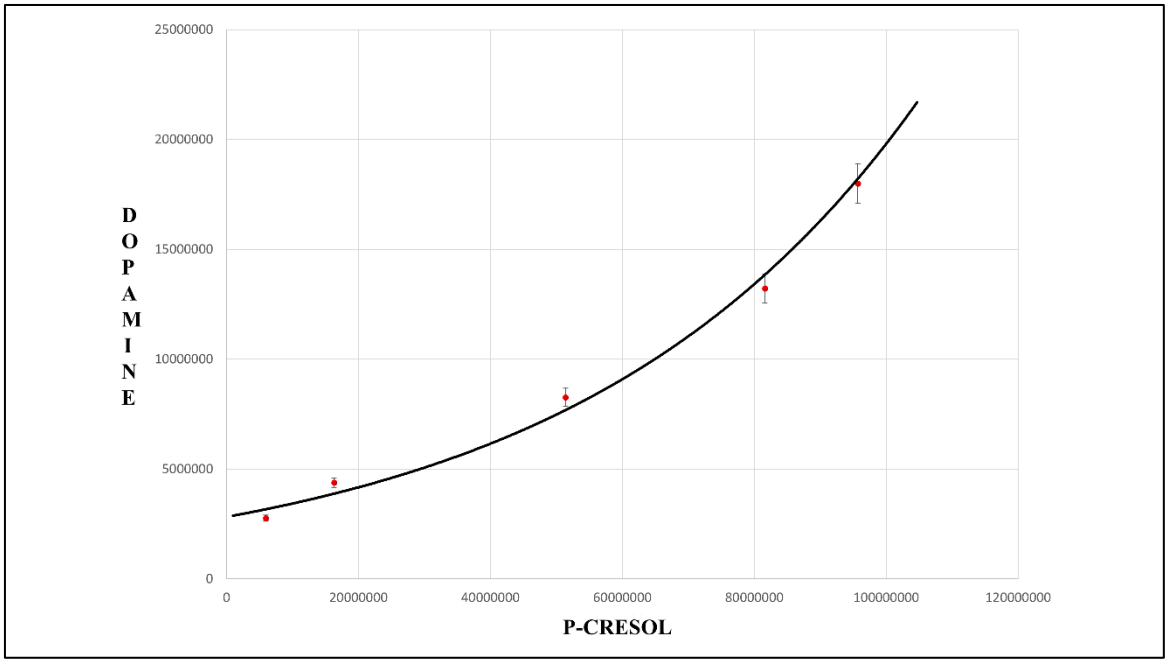


Figure 3.3: *Increase in concentration of p-cresol in relation to the accumulation of dopamine. Each dot represents a subset of autistic patients, divided by five p-cresol intensity ranges (First range: 1.00E+06–9.00E+06; Second range: 9.00E+06–3.50E+07; Third range; 3.50E+07–7.00E+07; Fourth range: 7.00E+07–8.50E+07; Fifth range: 8.50E+07–1.50E+08). The results represent the means ± SD of biological replicates.*

Thus, since it well known that 4-cresol, which we found higher in autistic subjects (Figure 3.4, Panel A), blocks dopamine β-hydroxylase (Southan et al., 1990), dopamine accumulation could be related to a its lower biotransformation into noradrenaline. In this regard, 4-cresol needs of higher concentration of ascorbic acid (vitamin C) to inhibit dopamine β-hydroxylase (Southan et al., 1990). Interestingly the concentration of latter resulted significantly higher (Figure 3.4, Panel B).

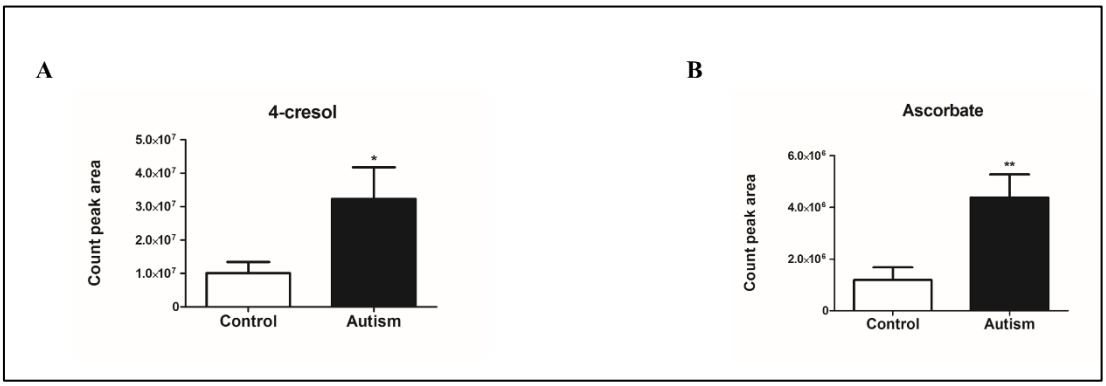


Figure 3.4: *(Panel A) Quantification of 4-cresol. The results represent the means ± SD of biological replicates. (Panel B) Quantification of vitamin C (ascorbate). Levels of vitamin C shows a slightly higher result in ASD subjects compared to controls this is required for inactivation ofβ-hydroxylase enzyme. *p < 0.05, **p < 0.01.*

Figure 3.5 confirmed that dopamine is not biotransformed into noradrenaline but prevalently into homovanillic acid (HVA), resulting higher. Accordingly, 3-methoxy-4-hydroxyphenethyleneglycol (MHPG), the first noradrenaline metabolite, was also found to be lower, as well as vanillylmandelic acid (VMA), the last metabolite of noradrenaline degradation (Figure 3.5).

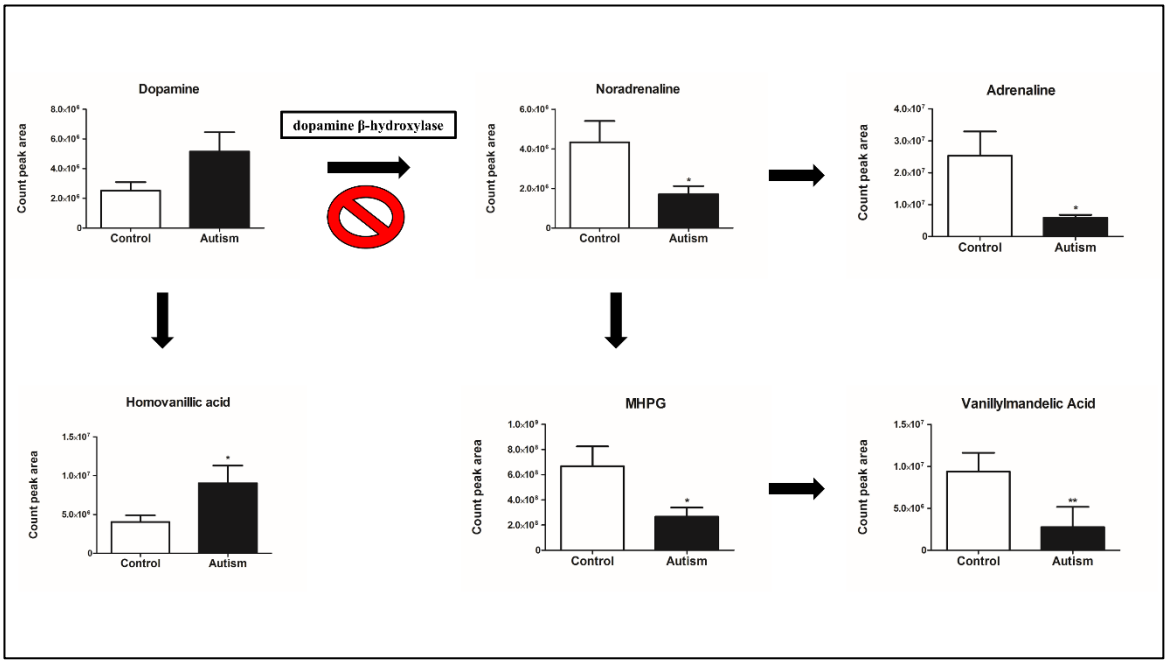


Figure 3.5: *Quantification of intermediates of dopamine and noradrenaline pathway and their metabolites. The results represent the means ±SD of biological replicates. *p<0.05, **p<0.01.*

Figure 3.6 showed lower levels of the active form of vitamin B6, pyridoxal phosphate (P5P), which is an essential cofactor for the biotransformation of glutamate into GABA explaining the lower P5P level recorded in ASD children and the higher level of glutamate as well as the lower concentration of GABA recorded (Figure 3.6).

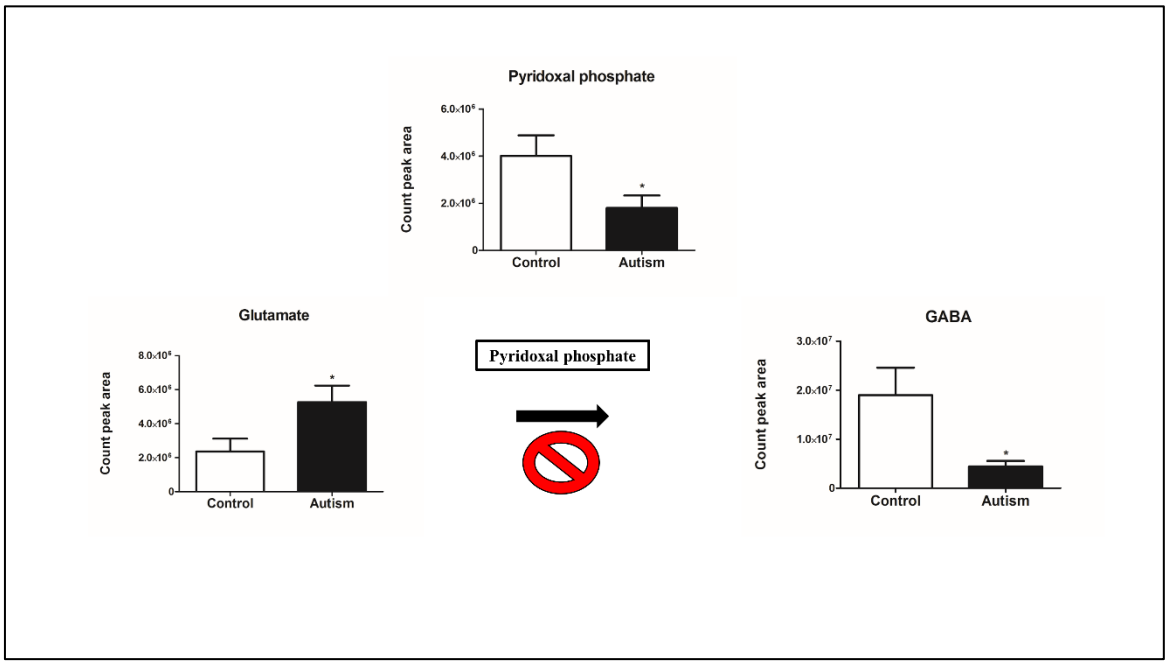


Figure 3.6: *Quantification of metabolites relative to excitatory/inhibitory neurotransmission is shown. A higher contraction of glutamate and a lower concentration of GABA. Peak areas for each metabolite were normalized by urinary specific gravity. The results represent the means ± SD of biological replicates. *p < 0.05.*

4. The concomitant lower concentration of vitamin B6, B9 and B12 may cause methylation deficency autistic children

4.1 Introduction

Among all autism spectrum diseases, autism is doubtless the most severe and can be distinguished from other neurodevelopmental conditions such as Asperger syndrome (AS) and the non-otherwise specified pervasive developmental disorder (PDD-NOS) (Levy et al., 2009), which shows specific peculiarities. Autism spectrum disorders (ASD) are increasingly recognized as a public health problem, which have increased in the last two decades from 2–5/10,000 to 1/68 children (Fombonne, 2009) (Wingate et al., 2014). Improvements in diagnostic criteria have contributed to this evident increase (Rutter, 2005). Several studies have shown the presence of alterations in neuropsychological function and of learning disabilities in subjects affected by metabolic alterations, which showed the presence of a progressive neuropsychological decline in areas of intellect, attention, memory and executive functions (Beauchamp et al., 2009). Recently, some metabolic abnormalities have been associated with various diseases involving behavioral disorders associated with autism. In fact, although autism spectrum disorder (ASD) is still diagnosed through behavioral observation due to a lack of laboratory biomarkers, recent metabolomics investigations on human biofluids such as urine and plasma (Cortelazzo et al., 2016) (Yap et al., 2010) provided a sensitive tool to identify metabolite profiles which could greatly aid clinicians in providing earlier and more reliable diagnoses.

A comparative analysis of the urine and plasma between ASD and control individuals showed that some metabolic pathways, most distinctive of young Italian autistic children, largely overlap with those found in rodent models of ASD, suggesting that autistic patients may share some metabolic abnormalities. Interestingly, changes in DNA methylation at differentially methylated CpG sites also correlated with total childhood autism symptom test scores (Piyathilake et al., 2011) (Wong et al., 2014).

Most of the urinary metabolites displaying the largest differences between young ASD and control children belonged to the tryptophan and purine metabolic pathways (Gevi et al., 2014). In ASD children, tryptophan is preferentially transmuted to xanthurenic acid and

quinolinic acid (two catabolites of the kynurenine pathway) at the expense of kynurenic acid and especially of melatonin. In addition, the gut microbiome contributes to altered tryptophan metabolism, yielding increased concentrations of indolyl 3-acetic acid and indolyl lactate. These results are consistent with the proposal of a purine-driven cell danger response, accompanied by overproduction of epileptogenic and excitotoxic quinolinic acid, large reductions in melatonin synthesis, and gut dysbiosis. In addition, vitamin B6, B12, riboflavin, phenylalanine-tyrosine-tryptophan biosynthesis, pantothenate and pyrimidine metabolism differed significantly in ASD children (Gevi et al., 2014) suggesting that these metabolic abnormalities could underlie several comorbidities frequently associated with ASD (Gevi et al., 2014). In this work, by using a more sensitive instrument (Orbitrap), allowing us to identify more metabolites, and extending our study to include a greater number of autistic children, we investigated the metabolic pathways that can be influenced by a simultaneous deficit of vitamin B6, vitamin B12 and folic acid (vitamin B9). Moreover, in this investigation, we used a targeted metabolomics platform and different samples in order to provide greater coverage of the metabolites than previous works (Gevi et al., 2014).

Vitamin B6 is the main cofactor of biological reactions such as the synthesis of neurotransmitters and trans-sulfuration, and it is also linked to the metabolism of tryptophan by transforming xanthurenic acid to nicotinic acid. There are three inactive conformations of vitamin B6 (pyridoxal, pyridoxine, pyridoxamine), which are converted into their biologically active form, pyridoxal 5′-phosphate (P5P). Low concentrations of P5P were found in autistic children (Adams et al., 2003) in combination with a low activity of the pyridoxal kinase enzyme which converts pyridoxal to P5P (Ranjan and Nasser, 2015). Vitamin B12 (cobalamin, Cbl) exists in multiple forms, including methylcobalamin (MeCbl) and adenosylcobalamin (AdoCbl), cofactors for methionine synthase (SM) and methylmalonylCoA mutase, respectively. The metabolically active form of vitamin B12, methylcobalamin (MeCbl) is an essential cofactor for the folate-dependent methylation of homocysteine to methionine by methionine synthase (MS) (Guéant et al., 2013) (Gherasim et al., 2013). Autism is associated with a lower vitamin B12 uptake that has been attributed to a dietary intake poor in vitamin B12, dysbiosis in the lining of the intestine that leads to poor absorption of B12, autoimmune antibodies, neurotoxin and heavy metal intoxication that make neurons insensitive to standard doses of B12 (Al-Farsi et al., 2013). High

concentrations of methylcobalamin are required to regenerate neurons and the myelin sheath of the spinal cord, which is necessary for the relief of symptoms in the autism disorder (Zhang et al., 2016). Folic acid (a form of vitamin B9) is a complex B vitamin that is similar to vitamin B12. Folates are involved in different reactions such as DNA synthesis and repair and in methylation pathways, in particular, it helps prevent the foetus from developing major congenital deformities of the brain or spine, including neural tube defects (Greenberg et al., 2011).

Results showed some altered pathways due to three vitamins deficits, including the transmethylation and trans-sulphurization pathways of methionine, justifying the well-known hypometilation of protein and DNA observed in autism (Hamza et al., 2017).

4.2 Materials and Methods

4.2.1 Participants

Sixty children with idiopathic ASD and 60 typically developing controls (aged 3–8 years) were recruited in Italy and northern Europe. The mean age (±S.D.) of cases and controls was 4.65±0.54 and 5.74±0.45 years, respectively, and the M:F ratio was 42:18 both in ASD and in controls. The diagnosis of autism was formulated according to the Diagnostic and Statistical Manual of Mental Disorders (5th edition) (American Psychiatric Association, 2013). The research is not a clinical trial and this procedure is in accordance with the Helsinki Declaration of the World Medical Association. Those analyses are considered exploratory.

4.2.2 Metabolites extraction, UHPLC-MS and data processing

The methods of urine collection, metabolite extraction, chromatography analysis and mass spectrometry were performed following the standard protocol listed in the previous paragraphs (3.2.2 and 3.2.3), as well as data processing and statistical analysis (paragraph 3.2.4).

4.3 Results

In our previous investigation, the urine samples of children were analyzed by using HILIC columns coupled to a mass analysis using a Q-Tof analyzer, while the statistical analysis was carried out with Metaboanalyst 3.0 software. In this investigation, we increased the number of individuals sampled, collected in different geographical areas, and used a more performant mass spectrometer (Orbitrap). Given that B vitamins are cofactors essential for various biological reactions related to autism, such as the synthesis of neurotransmitters, the trans-sulfuration process and the metabolism of tryptophan, we focused our attention on the concentrations of B vitamins and analyzed associated processes.

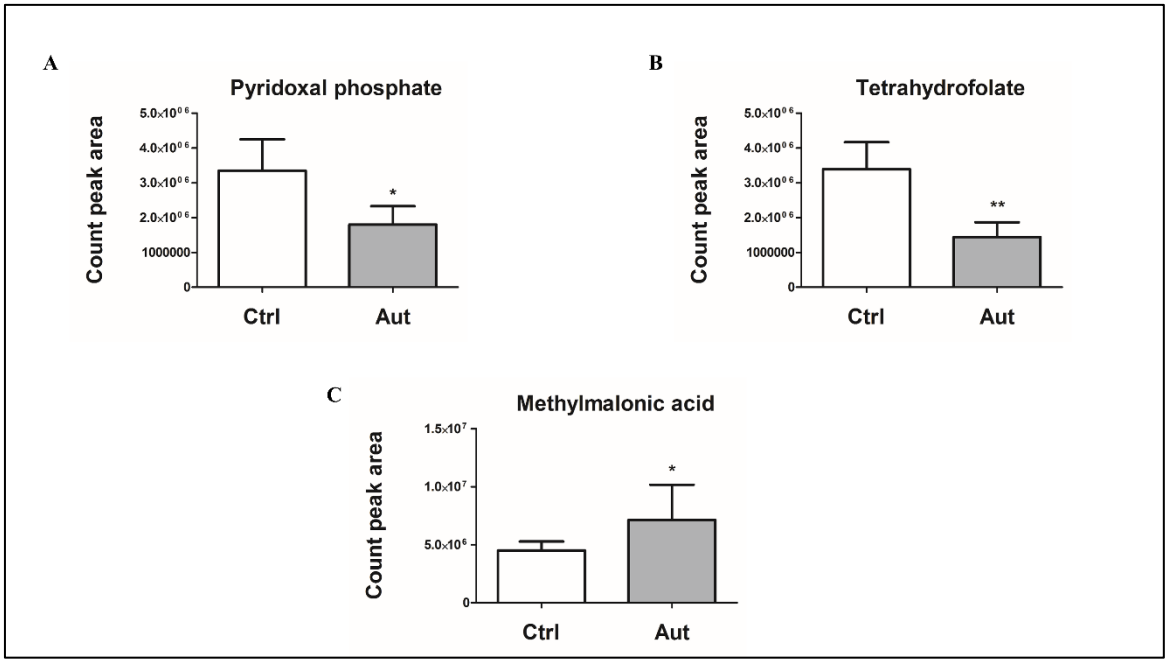


Figure 4.1: *Reliable indicators of vitamin B deficiency. Panel A reports pyridoxal 5'-phosphate (P5P), the active form of vitamin B6, that is involved in the trans-sulfuration process. Panel B reports tetrahydrofolate (THF), which is the final product of the folic acid cycle that participates in the folate-dependent methylation of homocysteine. Panel C reports methylmalonic acid (MMA), which in the urine test is the most reliable method for determining vitamin B12 deficiency. The results represent the means ± SD of biological replicates *P < 0.05, ** P < 0.01.*

Figure 4.1 reports the values of reliable indicators of vitamin B deficiencies measured in the urine from autistic children. Pyridoxal 5′-phosphate, the active form of vitamin B6 (Figure 4.1A) and an enzyme cofactor involved in the trans-sulfuration process, was altered in the urine from autistic subjects. In the case of vitamin B9, the amount of tetrahydrofolate was reported (Figure 4.1B) because it is the final product of the folic acid cycle, which participates in the folate-dependent methylation of homocysteine. Finally, the values of methylmalonic acid (MMA) were determined to assess the state of vitamin B12 (Figure 4.1C), which is the most reliable urine test for determining vitamin B12 deficiency. High MMA values are indicative of vitamin B12 deficiency (Robert and Brown, 2003).

Regarding the altered metabolomics pathways (Figure 4.2), the active form of vitamin B6 (P5P) is a fundamental cofactor of cystathionine β-synthase enzyme (CBS) in the bioelimination of toxic homocysteine in the trans-sulfuration process. A low cystathionine concentration in autistic children accounts for the accumulation of homocysteine and serine (upside of Figure 4.2) in these patients.

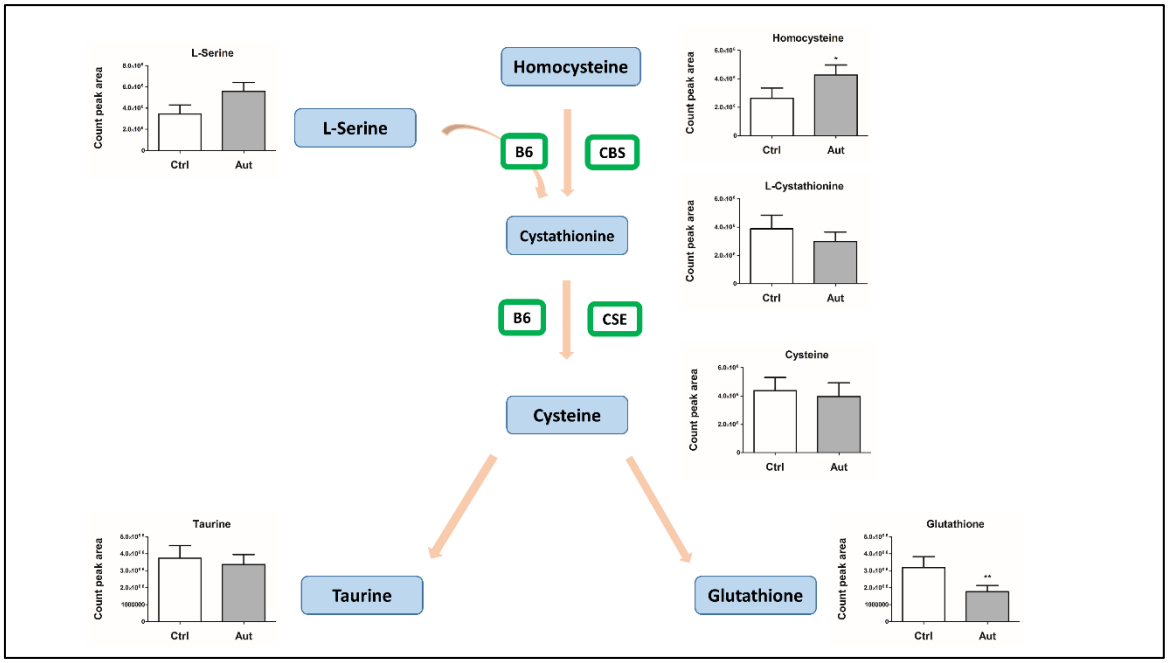


Figure 4.2: *Quantification of the trans-sulfuration pathway. Vitamin B6 deficiency prevents the correct functioning of the enzyme cystathionine β-synthase (CBS) inducing the accumulation of homocysteine and serine and a reduction in cystathionine concentration in autistic children. Peak areas for each metabolite were normalized by urinary specific gravity. The results represent the means ± SD of biological replicates *P < 0.05, ** P < 0.01.*

As with cystathionine, less cysteine is produced; it also requires vitamin B6 again as a cofactor of the cystathionine γ-lyase enzyme (CSE) for its production. Finally, lower concentrations of taurine as well as glutathione (GSH) were detected in the autistic patients, which may result in less protective action in oxidative processes. Homocysteine, in addition to its irreversible bio-elimination by trans-sulfuration, can be re-methylated to methionine (Figure 4.3).

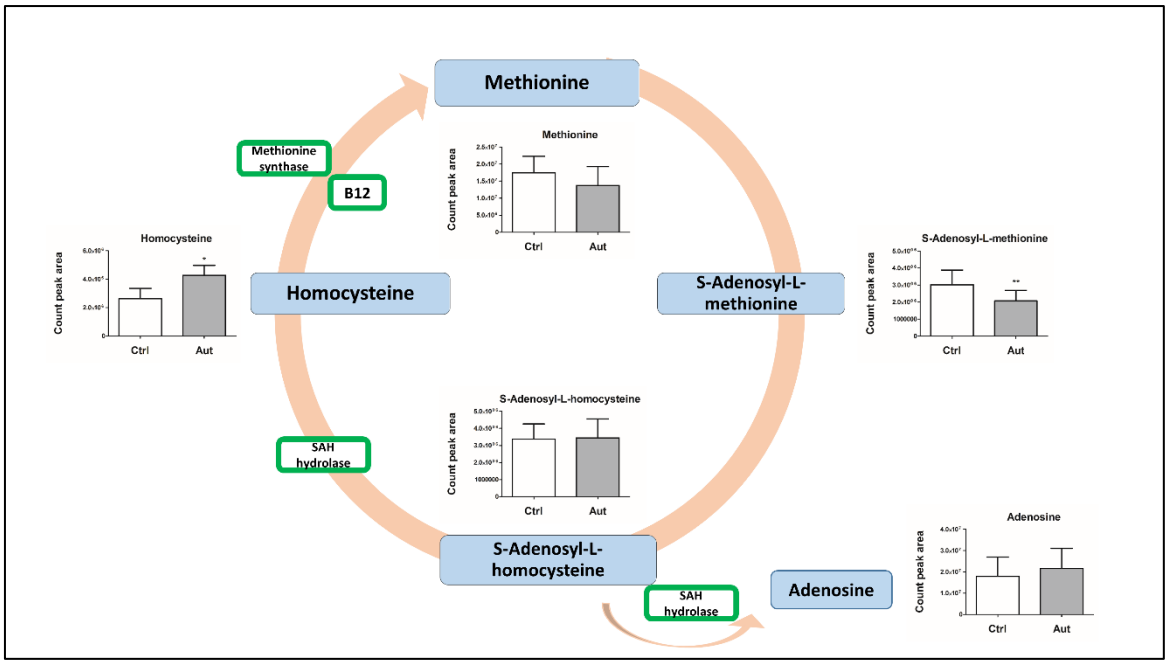


Figure 4.3: *Quantification of metabolites of methionine cycle. Vitamin B12 deficiency that acts as a cofactor of methionine synthase (MS) induces low S-adenosyl-L-methionine (SAM) concentration caused by a methyl group deficiency. Peak areas for each metabolite were normalized by urinary specific gravity. The results represent the means ± SD of biological replicates *P < 0.05, ** P < 0.01.*

Thus, the reduced presence of vitamin B12 in autistic patients blocks this transformation to methionine, which is consistent with the low concentrations recorded in this study. Methionine's methyl group is activated by ATP to form S-adenosyl-L-methionine (SAM), which is a methyl donor for many reactions. A block in the formation of methionine will result in a methyl group deficiency that will prevent SAM formation; this justifies the lower concentrations of SAM in autistic patients. Normally the removal of the methyl group from SAM would lead to the formation of S-adenosyl-L-homocysteine (SAH), which would be

immediately converted to homocysteine by the enzyme SAH hydrolase (SAHH), with the removal of the adenosine molecule. This step cannot occur in cases of SAM deficiency. In fact, the concentrations of SAH and adenosine are higher in autistic children. Since the conversion of SAH to homocysteine is reversible, with the equilibrium favoring the formation of SAH, increases in urine homocysteine are matched by an elevation of SAH in most cases. Finally, since vitamin B12 is also involved in the folate cycle (Figure 4.4A), the donation of a methyl group from 5-methyltetrahydrofolate (5-methylTHF) to tetrahydrofolate (THF) is strongly reduced, justifying the reduced production of THF recorded in our analysis and the accumulation of 5-methyl-THF. In addition, the concentrations of choline and betaine, the latter of which is transformed into N-N-dimethylglycine (Figure 4.4B), functional for the re-methylation of homocysteine, have been altered. Therefore, the concomitant alteration of these metabolic pathways in autistic children significantly reduces the availability of methyl groups and consequently alters the methylation processes.

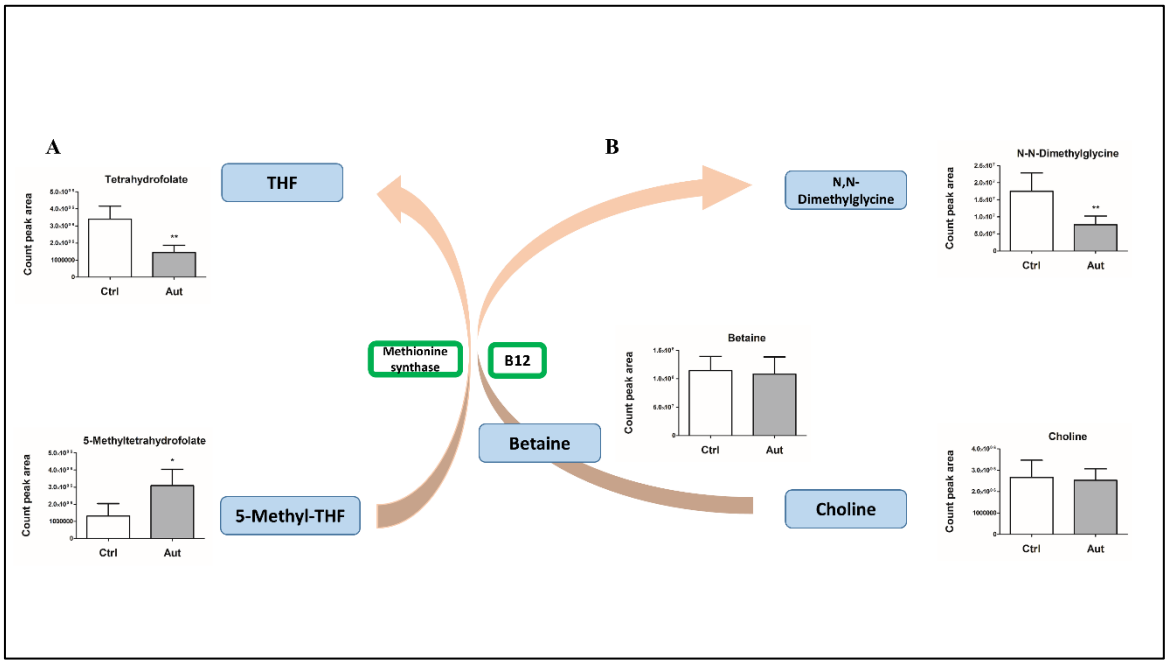


Figure 4.4: *Panel A shows quantification of the main metabolites included in the folate pathway. Deficiency of vitamin B12 induces a blockage in the folate cycle carrying an accumulation of 5-methyltetrahydrofolate (5-methylTHF). Panel B shows the metabolites involved in the transformation of betaine to N-N dimethylglycine. Peak areas for each metabolite were normalized by urinary specific gravity. The results represent the means ± SD of biological replicates *P < 0.05, ** P < 0.01.*

Finally, Figure 4.5 shows a broader picture of the pathways altered in autistic children as related to vitamin B deficiencies. The decreased methionine cycle turnover, related to reduced SAM synthesis, is concomitant with a reduction in cysteine and glutathione synthesis and the two phenomena correlate with three vitamin B deficiencies. In fact, the activation of methionine synthase (MS) requires vitamin B12 as a cofactor, while inhibition of SAH hydrolase activity is induced by oxidative stress resulting from vitamin B6 deficiency. Obviously, the consistent decrease in the SAM / SAH ratio is related to a lack of vitamin B6.

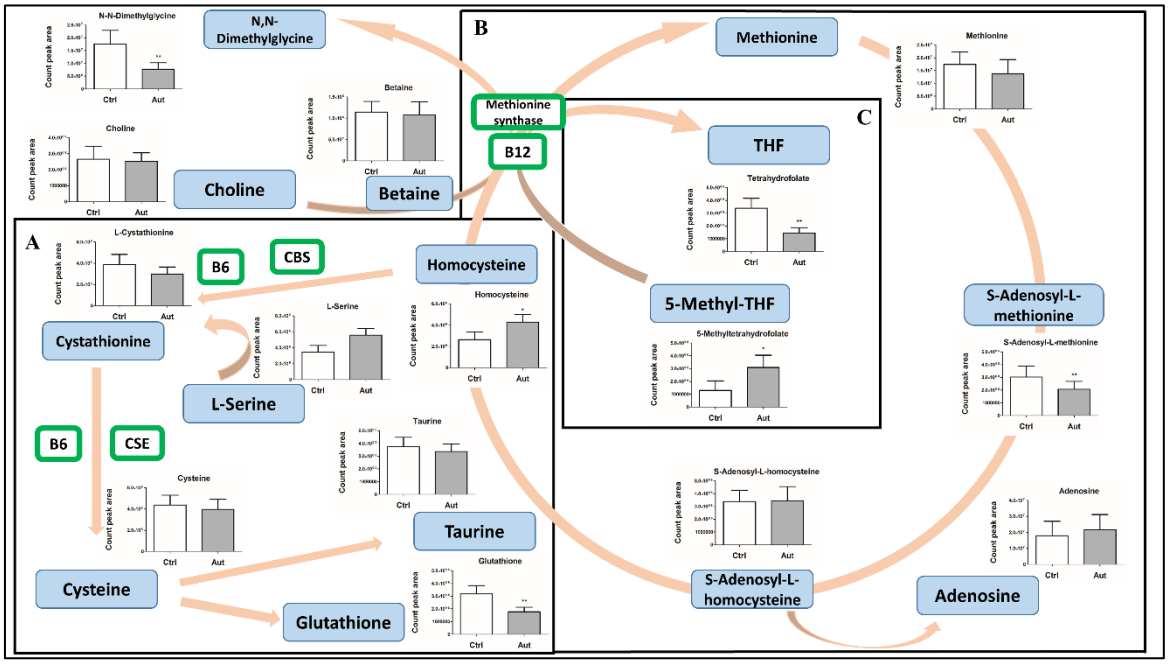


Figure 4.5: *Overview of the previously discussed cycles. Panel A represents the trans-sulfuration process in which homocysteine is eliminated and transformed into antioxidant precursors such as glutathione (GSH). Panel B shows the methionine cycle essential for the methylation process with the formation of S-adenosyl-L-methionine (SAM) as the main methyl donor. Panel C shows folate cycle: 5-methyltetrahydrofolate (5-methylTHF), which cannot provide the methyl group that is essential for the methylation reaction. The values were obtained from the urine of 60 autistic children compared to 60 matched controls (age range 3–8 years). The results represent the means ± SD of biological replicates * P <0.05, ** P <0.01.*

5. Discussion

Recently, there has been growing interest in identifying which metabolic pathways are affected in autism (Gevi et al., 2016; Belardo et al., 2019). In the first part of this thesis, by an accurate metabolomic analysis, we identified more differently expressed metabolites in the urine of autistic subjects respect to controls, in particular those involved in neurotransmission, production or biotransformation. Data analysis showed a higher levels of dopamine and its metabolite, homovanillic acid, probably along with reduced dopamine β-hydroxylase enzyme activity, a key enzyme in catecholamine metabolism and responsible for converting dopamine to noradrenaline (Southan et al., 1990). Further, our metabolomic analysis showed that the accumulation of dopamine in the urine of autistic subjects increases with the increase of 4-cresol, in agreement to Pascucci et al. (2020). 4-Cresol is produced only by bacteria and not by human cells (Saito et al., 2018), which are not able to methylate tyrosine into 4-cresol. 4-Cresol was found high even though autistic children were fed tyrosine-poor diets (Altieri et al., 2011). 4-Cresol or similar phenols, are able to insert a radical into an active residue site of tyrosine at position 216 of the enzyme, leading to the irreversible inactivation of dopamine β-hydroxylase. For this chemical reaction, phenols require the presence of vitamin C (ascorbate) (Southan and DeWolf, 1990), which resulted higher, in agreement to Steinert et al. (2020). 4-Cresol is produced by several bacteria, but specifically by *Clostridium difficile*, which has been found by many authors in the intestine of ASD children (Argou-Cardozo and Zeidán-Chuli, 2018). For these reasons, some authors proposed to use the presence of Clostridium difficile and/or presence of 4-cresol as a biomarker for the diagnosis of autism (Li et al., 2017). Hereby prove, it is advisable to reduce the presence of *Clostridium difficile* and the administration of vitamin C in autistic children in order not to exacerbate these chemical reactions. Figure 5.1 summarizes the cascade of events reported in this work that starts from the presence of significant amount of 4-cresol which in the presence of high vitamin C (ascorbate) causing the block of dopamine β-hydroxylase enzyme, accumulation of dopamine and homovanillic acid, but lower noradrenaline and adrenaline production, as well as lower MHPG and vanillylmandelic acid.

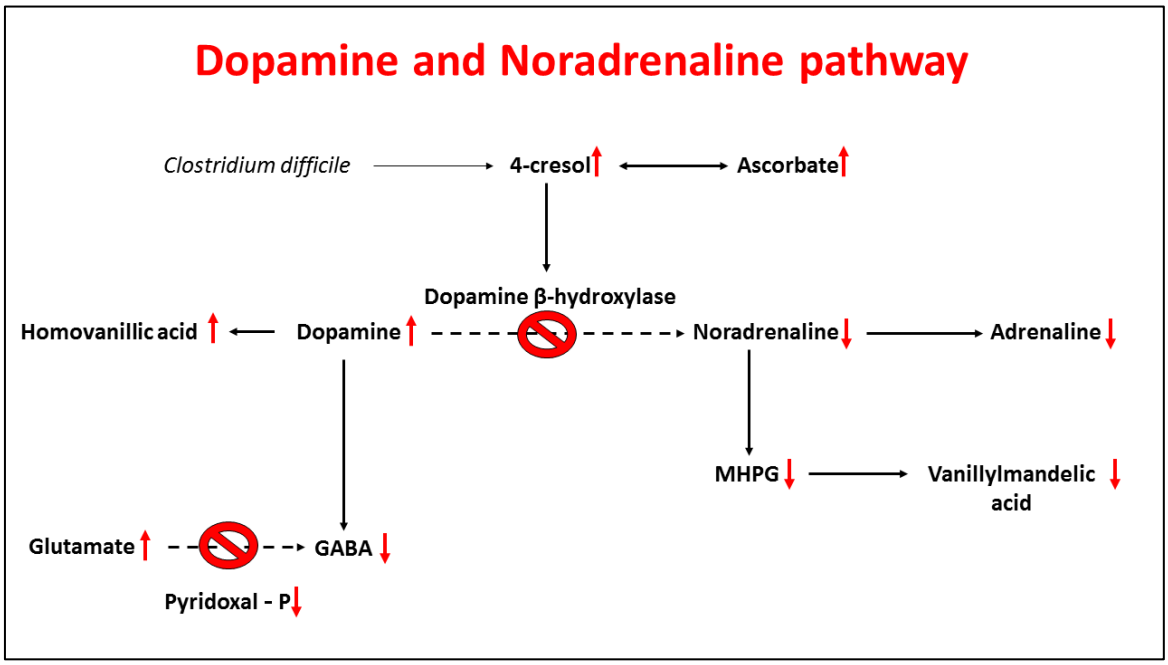


Figure 5.1: *Overview of noradrenaline and adrenaline degradation pathway.*

Generally, the accumulation of dopamine is toxic (Ben-Shachar et al., 2004) and an excess has been implicated in the aetiology of psychotic behaviour and schizophrenia (Brisch et al., 2014). In fact, high dopamine levels are related to numerous deficits and associated symptoms in ASD, including convulsions (Starr, 1996; Tuchman and Rapin, 2002), motor dysfunction (Rinehart et al., 2001; Rinehart et al., 2006), stereotyped behaviours (Canales and Graybie, 2000; Ralph-Williams et al., 2003) and neurogenesis (Prior and Hoffman, 1990). In agreement, drugs that inhibit dopamine binding to dopamine receptors have been widely used as antipsychotic drugs and have been applied in the treatment of autism (Posey et al., 2008). In addition, in ASD subjects, dopamine is not converted into noradrenaline but into homovanillic acid (HVA), which was found significantly increased (Figure 3.5). On the other hand, there is a link between the severity of the symptoms of ASD and the concentration of HVA; in fact, a relationship between the concentration of urinary HVA and increased agitation, stereotyped behaviours and reduced spontaneous behaviour was recently reported (Kałuzna-Czaplińska et al., 2010). In support of this, other studies have shown that vitamin B6 supplementation, which from our metabolomic analysis is lacking in autistic children, reduced the concentrations of urinary HVA and significantly reduced

neurological symptoms (Lelord et al., 1978). Higher levels of dopamine are also responsible of the reducedreleaseofγ-aminobutyricacid (GABA). In fact, dopaminebinds two types of receptors, D1 and D2: at low concentrations, dopamine interacts with D1 and stimulates the production of GABA (Bernath and Zigmond, 1989), whereas at higher concentrations, it binds to D2 receptors and exerts an inhibitory influence on the release of GABA (Floran et al., 1997; Harsing and Zigmond, 1997). Recently, an association between significant anomalies in the nervous circuits and lower GABA levels was found (Robertson et al., 2016). Lower GABA levels are also consequent to the decreased availability of P5P (vitamin B6), as recorded here in ASD subjects, since the biotransformation of glutamate into GABA requires P5P as a cofactor of the enzyme glutamate decarboxylase (GAD) (Sato, 2018). Vitamin B6 deficiency can lead to various metabolic imbalances in ASD children, in particular hypomethylation of DNA (Belardo et al., 2019). Thus, both the absence of P5P and higher levels of dopamine contribute to reduce GABA concentration, while glutamate accumulates. On the other hand, high levels of glutamate can cause nervous excitement, creating neurological inflammation and other damage, as observed in autism (Moghaddam and Javitt, 2012). According to clinical studies, high glutamate levels contribute to the development of a series of neurodegenerative diseases, such as Huntington's disease, Parkinson's disease, Alzheimer's disease and multiple sclerosis (Lewerenz and Mahe, 2015), although in children, adequate levels of glutamate are essential for learning, as well as for the functioning of short- and longterm memory (Moghaddam and Javitt, 2012). As mentioned above, the inhibition of dopamine β-hydroxylase significantly reduces the levels of noradrenaline and adrenaline as well as of their two products: 3-methoxy-4-hydroxyphenyleneglycol (MHPG) and vanillylmandelic acid (VMA) (Figure 3.5). MHPG, the main brain metabolite of noradrenaline excreted in urine, is one of the markers for different mental disorders (Kurita et al., 2015) and its lower levels suggest reduced activity of the noradrenergic system (Young et al., 1978). Moreover, the lower levels of noradrenaline and adrenaline reflect an adaptive response of ASD changes in the continuous state of anxiety through the damping of noradrenergic activity (Young et al., 1978) as well as a lack of attention, and difficulty in learning and memory in autistic children (Klinger and Dawson, 2001). From what has been reported it is clear that in autism the dynamic equilibrium between neurotransmitters is altered. There are, in fact, two types of neurotransmitters: excitatory ones (such as glutamate), which stimulate, and

inhibitory ones (such as serotonin or GABA), which sedate the nervous system to balance the mood. It may happen that an excitatory neurotransmitter becomes predominant over the partner of the inhibitory neurotransmitter, which should counterbalance the effect, thereby increasing nervous stress. On the contrary, if the inhibitory partner is excessively dominant, there is a state of somnolence, lethargy or even depression. Thus, it is not surprising that an imbalance between excitatory/inhibitory neurotransmission is a critical point of the neurobiology of autism (Rubenstein and Merzenic, 2003), causing a concomitant increase with the prevalence of convulsions among individuals with ASD (Canitano, 2007; 2017). Clearly, the cascade of events reported in Figure 5.1 represents a classic example of the "microbiota-brain-gut axis" (Sampson and Mazmanian, 2015; Bienenstock et al., 2015; Mayer et al., 2015), since it is related to the presence of 4-cresol in autistic children, specifically produced by *Clostridium difficile*, as consequence of an altered microbiota. Many studies of faecal DNA extracts have found a greater presence of *Clostridium* or *Desulfovibrio* clusters in children with gastro-intestinal disorders and ASD than in children with only similar gastro-intestinal disorders but with normal neuro-behavioural development (Finegold et al., 2011; Parracho et al., 2005; Song et al., 2004). The "brain-gut-microbiota axis" allows the microbial population of the gut to communicate with the brain and vice-versa (Rhee et al., 2009), suggesting a change in basic neuroscience assumptions (Mayer et al., 2014). Intestinal dysbiosis at an early or late age, can have a profound impact on brain function, although the mechanisms of signal transmission are complicated and not fully explained (El Aidy et al., 2015; Grenham et al., 2011). Recent reports documented in autistic children the presence of chronic inflammation, an increased predominance of gastrointestinal inflammation and increased mucosal permeability in the upper and lower intestines (White, 2003) that may be mediated by innate microglial activation and proinflammatory cytokines (Vargas et al., 2005). The inflammatory response enhances when GSH concentrations are low (Pietro, 2011), as found in our previous investigation (Gevi et al., 2016). Furthemore, chronic inflammation decreases GSH which promotes a self cycle that could exasperate gastrointestinal and central nervous system inflammation related with autism. Thus, it is generally accepted that the increased permeability of the intestinal tract in ASD individuals, known as "intestinal permeability", is the fundamental factor underlying the relationship between ASD and the gut (Mangiola et al., 2016). In agreement, ASD animals with gastrointestinal barrier deficiencies allowed

toxins and bacterial products to enter into the bloodstream, affecting brain function (Hsiao et al., 2013). Gut bacteria may also produce their own neurotransmitters, which simulating those of the host and may compromise host neural pathways, such as serotonin, GABA, noradrenaline and dopamine (Li et al., 2017; Barrett et al., 2013), as reported in this thesis. In support, raising animals in the absence of microbial colonization causes behavioural anomalies, supporting that the microbiota modulates behavioural outcomes in animal models (Helen et al., 2017).

Referring to figure 3.2 it is easy to see that one of the most altered pathways is that of pyridoxal phosphate (vitamin B6). As already found in the first analyses, a deficiency of the active form of vitamin B6 is present in ASD children. For this reason, in the second part of this thesis, significant urinary metabolic differences have been reported between healthy children and those with idiopathic ASD, linked to reduced concentrations not only of vitamin B6, but also of folic acid (vitamin B9) and vitamin B12, which can explain the alteration of proteins and DNA methylation of autistic children, reported in the literature (Hamza et al., 2017; Nardone et al., 2014).

It is interesting to note that dietary vitamin B deficiencies complicate the care of critically autistic children (Baird and Ravindranath, 2015). Using a larger number of samples it was possible to obtain a picture of the altered pathways in autistic children in relation to vitamin B deficiencies (Figure 4.5). Pyridoxal 5′-phosphate (P5P) is the active form of vitamin B6 that is used as a cofactor, together with the enzyme cystathionine β-synthase, for the condensation of serine and homocysteine to form cystathionine. This is further converted to cysteine by completing the process called trans-sulfuration, which again requires vitamin B6 as a cofactor of the enzyme cystathionine γ-lyase (CSE) (Kern et al., 2011). Thus, lower concentrations of vitamin B6 block cysteine formation and result in the accumulation of the toxic homocysteine (Figure 4.5A). The consistent decrease in plasma methionine and cysteine concentrations observed in autistic children suggested that cysteine may be an essential amino acid for these children (James et al., 2009). More importantly, cysteine is the source of both taurine and hydrogen sulfide (H2S), in addition to being the essential amino acid constituent in the functional (CXXC) motif of the major cellular antioxidant families, which includes reduced glutathione (GSH). Since the intracellular GSH-GSSG redox status provides the essential intracellular reducing environment required for normal

immune function, detoxification capacity, redox-sensitive enzyme activity, and membrane redox signaling (Schafer and Buettner, 2001; Reid and Jahoor, 2001; Biswas et al., 2006) it is not surprising that autistic children exhibit systemic evidence of oxidative stress. Oxidative stress occurs when antioxidant defense mechanisms fail to counterbalance and control reactive oxygen species generated from endogenous oxidative metabolism or from pro-oxidant environmental exposures, such as vitamin B6 deficiency. Several recent reviews and research studies lend support to the hypothesis that redox imbalance and oxidative stress may be a contributing factor to autism pathology (James et al., 2004; Chauhan and Chauhan, 2006; Parker et al., 2017). This theory has sparked a new field of research focused on optimizing glutathione concentrations as a way to treat autism (Kern et al., 2011). In agreement, a decrease in glutathione concentrations was also recorded in children with autism but consequent to increased environmental mercury concentrations, bad gut bacteria, and poor diet (Chauhan and Chauhan, 2006), supporting that GSH reduction may result from vitamin B6 deficiency. Paşca presented a possible connection between autism spectrum disorders (ASD) and high concentrations of homocysteine for the first time in 2006 (Paşca et al., 2006). Recent studies have confirmed that high concentrations of homocysteine in biological fluids are related to oxidative stress and are generally associated with neuropsychiatric disorders such as autism (Chauhan and Chauhan, 2006; Kałużna-Czaplińska, 2013). Homocysteine leads to increased excitatory glutamatergic neurotransmission in different areas of the brain where neuronal damage results from excess Ca^{2+} influx and reactive oxygen generation (Ho et al., 2002), which results in increased oxidative stress. Thus, vitamin B6, B12 and B9 deficits act in a syntonic manner. Furthermore, various studies have suggested that homocysteine might regulate the function of other neuromodulators, such as acetylcholine (Chen et al., 2001) dopamine and serotonin (Gao et al., 2011), in agreement with our previous investigations of altered tryptophan metabolism in autism (Gevi et al., 2016). These results further support the idea that more than one metabolomics pathway is altered in autism, which may not be correlated to concomitant specific gene mutations. The accumulation of homocysteine is also a consequence of vitamin B12 deficiency (Figure 4.5B) (Gilfix, 2005) and its high levels were associated with oxidative stress and DNA hypomethylation (Jiang et al., 2007). The re-methylation of homocysteine to methionine requires a methyl group provided by 5-methyltetrahydrofolate, through methionine synthase (MS) and the cofactor vitamin B12

(Blom et al., 2006). The reduced activity of MS leads to lower concentrations of tetrahydrofolate (folate cycle), as reported in Figure 4.5C. A deficit of methionine synthase or a deficiency of B12 may lead to a so-called "methyl-trap" of tetrahydrofolate (THF), in which 5-methylTHF is not converted into THF, which does not provide methyl groups for methylation reactions (Hoffbrand and Weir, 2001). The strict relationship between folic acid and vitamin B12 is shown in Figure 4.5C. For this reason, folate and vitamin B12 deficiencies have been implicated in the manifestation of various clinical disorders (Al-Maskari et al., 2012) and have been shown to arrest children's development (Shakur et al., 2010). Children with autism or a pervasive developmental disorder very often exhibit feeding difficulties (Ahearn et al., 2001). Although folate is required for metabolic processes and neural development, there is a debate about its protective effect against ASD. Some authors have concluded that a folic acid deficit is associated with an increased risk for autism, however higher concentrations can give negative neurocognitive development outcomes (Darrell and De Soto, 2017). Transformation of homocysteine to methionine requires vitamin B12 as a cofactor of methionine synthase. Since the activity of methionine synthase determines the relationship between S-adenosyl-L-methionine (SAM) and the methylation inhibitor S-adenosyl-L-homocysteine (SAH), vitamin B12 deficiency can influence hundreds of SAM-dependent methylation reactions (Zhang et al., 2016). There was a significant decrease in urinary methionine and S-adenosyl-L-methionine (SAM) concentrations, which is the major methyl donor, while adenosine and S-adenosyl-L-homocysteine (SAH) concentrations increased. Low concentrations of methionine and SAM in combination with increased concentrations of SAH and adenosine have been previously demonstrated to be associated with reduced cellular methylation (Yi et al., 2000). In particular, a decrease in the SAM / SAH ratio has been associated with hypomethylation of DNA, RNA, proteins, phospholipids and neurotransmitters (Finkelstein, 2007) exerting an important epigenetic effect. Thus, our investigation and reconstruction of altered metabolic pathways we shed light it may be one possible explanation of the relationship between ASD and DNA hypomethylation (Piyathilake et al., 2011; Wong et al., 2014). In this regard, we are currently investigating the correlation between the decreased SAH/SAM ratios recorded in subgroup of autistic children with the degree of DNA methylation recorded in their lymphocytes (data not shown). This will better validate what previously reported by other authors (Piyathilake et al., 2011; Wong et

al., 2014) that the decreased methylation process, related to vitamin B deficiencies, results in hypomethylation of DNA in autism.

What is common in most autistic patients is a presence of intestinal dysbiosis associated with chronic inflammation, an increased prevalence of gastrointestinal inflammation and increased mucosal permeability in the upper and lower intestines (White, 2003) that may be mediated by innate microglial activation and proinflammatory cytokines (Vargas et al., 2005). The inflammatory response is increased when GSH concentrations are low (Pietro, 2011), as here recorded. Moreover, chronic inflammation depletes GSH, which further promotes a self-perpetuating cycle that could exacerbate gastrointestinal, central nervous system inflammation, associated with autism, and exacerbate oxidative stress (White, 2003).

6. Conclusions

In recent years, the number of researches relating to the study of the relationship between intestinal flora and brain development has increased. The hypothesis that an alteration in the composition of the microbiota activity can be related to the autism spectrum disorder is supported by the fact that half of people with autism have gastrointestinal problems associated with an alteration of the permeability of the intestinal barrier. Microbial colonization affects mammalian brain development in the first years of life and adult behavior in the later stages of growth. It is likely that genetic predispositions and environmental influences can affect the composition of the normal intestinal microbiota, predisposing the child to potential states of acute and chronic inflammation up to the onset of real diseases. Scientifically it is increasingly evident that there is a complex system of relationships that binds the central nervous system, the intestinal tract and the microbiota. This is known as the "microbiota - gut - brain axis" and results of fundamental importance for maintaining the homeostasis of the human organism with the microorganisms of the intestinal bacterial flora that seem to play an important role. P-cresol, produced by intestinal bacteria, inhibits dopamine beta-hydroxylase, representing a classic example of this axis. In addition, an increase in excreted metabolites derived from the microbiome was found in the urine of autistic children, which support the thesis of intestinal dysbiosis commonly associated with ASD (Gevi et al., 2016). A key factor underlying the relationship between ASD and gut is the increased permeability of the intestinal tract in ASD individuals, known as "leaky gut". Previous studies have shown that animals with ASD have defects in the gastrointestinal barrier, leading to the entry of toxins and bacterial products into the blood, which affect brain function. Gut bacteria can also interact by producing a series of their own neurotransmitters, which mimic those of the host and thus alter neural pathways, such as GABA, norepinephrine, serotonin and dopamine.

In this PhD thesis it was demonstrated how the documented presence of an altered microbiome can negatively affect the metabolism of ASD patients. The altered microbiome does not allow a correct absorption of the B vitamins which in turn causes negative effects on the organism, such as oxidative stress or DNA hypomethylation. Similarly, intestinal

dysbiosis involves the production of toxic metabolites for the patient, such as p-cresol, which significantly impairs neuronal transmission.

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