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Epigenetic analyses in Autism Spectrum Disorders: gender differences and the contribution of maternal risk factors

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ABSTRACT

ASD are one of the largest groups of complex neurodevelopment disorders that affect about 1-2% of the population with a greater frequency in males, in a ratio of 4.5: 1. The diagnosis of ASD is made on the basis of the clinical observation of the subject and the use of standardized assessment scales, such as ADOS and ADI-R. In recent decades, the prevalence of this disorder has increased significantly and this is thought to be due to both a better understanding of the problem and an improvement in the process and diagnostic criteria. Although numerous studies carried out to date show a considerable variety of causes that can lead to the development of ASD, the aetiology of this disorder still remains unknown but it has been shown that environmental, genetic and epigenetic factors play an important role.

In the present study we investigated, in a population of 42 girls affected by ASD, the correlation between the levels of methylation of genes associated with this disorder, maternal risk factors and symptomatological severity. To this end, the experimental protocols for the analysis of 7 genes, namely *MECP2*, *OXTR*, *BDNF*, *5-HTR1A*, *RELN*, *BCL-2*, and *EN2,* were developed using the Methylation Sensitive-High Resolution Melting technique. The anamnestic data were collected through the administration of a questionnaire to the mothers on their lifestyle before and during pregnancy, while the symptomatological severity of the ASD girls were evaluated using the "gold standard" ADOS-2 psychodiagnostic tool. We also recruited 25 ASD boys as comparison population, in order to assess the presence of any differences between the two genders. The results obtained from the methylation analyses showed that, except for the *MECP2* promoter, all the other investigated genes showed very low methylation levels, of about 1-2% in average. However, three of the analyzed genes, namely

MECP2, OXTR and *RELN*, showed significant differences in mean methylation levels between males and females. The methylation levels found were subsequently correlated with maternal factors extrapolated from the questionnaire, and these correlations revealed a statistically significant association between *BDNF* gene methylation levels and weight gain in pregnancy. Finally, we made a correlation between maternal factors and symptomatological severity finding a statistically significant association between ASD severity and lack of folic acid intake during pregnancy. These results could suggest a role of epigenetic modifications and maternal factors in the aetiopathogenesis of ASD and therefore further studies in this sense could allow a better understanding of the importance of these factors in the pathogenesis of these disorders.

INTRODUCTION

Autism, from the Greek *autós*, means itself. Autism Spectrum Disorders (ASD) are one of the largest groups of complex neurodevelopmental pathologies defined by 3 clinical features: (1) impairment in social interaction; (2) impairment in communication; and (3) restricted and repetitive patterns of behavior and interests [1]. ASD is known as a "spectrum" disorder because it is characterized by wide variability in the type and severity of symptoms that can occur. This causes two individuals "on the spectrum" to appear very different from each other despite being classified under the same diagnostic umbrella. The term 'autism' was introduced at the beginning of the XX century by Bleuler to indicate a behaviour, which is observed in schizophrenic patients, characterised by closure, avoidance of the other and isolation. Subsequently, Leo Kanner (1943) coined the term Childhood Autism to describe a set of characteristics found in two groups of children. All the children studied by Kanner, in the study published in the journal Nervous Child (1943), showed a relational absence, and significant deficits in communication and language. Kanner defined them as suffering from “Autistic Disturbances of Affective Contact”. He pointed out in these children the importance of routines that led them to limit gestures and movements by concentrating them on repetition and named this condition “Early infantile autism”, which is now known as autism. In 1944, Hans Asperger, an Austrian pediatrician, described patients with similar characteristics of impaired communication and social interaction to those studied by Kanner. However, most of these conditions were milder forms of autism and included a disability in both motor and speech ability. These symptoms will be used later to define Asperger's syndrome.

In 1952 the American Psychiatric Association (APA) publishes the first edition of the DSM (Diagnostic and Statistical Manual of Mental Disorders) (DSM-I) that defines autism as a psychiatric illness: a form of childhood schizophrenia characterized by a detachment from reality. Subsequently autism was thought to be due to the behaviour of cold, emotionless mothers, which Bruno Bettelheim called "refrigerated mothers". This concept was denied in the 1960s and 1970s, as a growing body of research showed that autism was biologically based and closely related to brain development. The DSM-III, published in 1980, described autism as a "pervasive developmental disorder" distinguishing it from schizophrenia. DSM-III listed for the first-time specific criteria required for a diagnosis:

- lack of interest in people
- severe communication impairment
- bizarre responses to the environment.

All these had to occur within the first 30 months of life. The DSM-IV, published in the 1990s, was the first edition of the DSM to classify autism as an autism spectrum. This version listed five conditions with distinct characteristics: autism, pervasive developmental disorder, Asperger's disease, Disintegrative Disorder of childhood (CDD) and Rett's syndrome. Furthermore, a very important aspect was that autism was mainly linked to genetic causes. In 2013 the fifth edition of the DSM (DSM-V) is published introducing the term "Autism Spectrum Disorder" to define a diagnosis characterized by two groups of characteristics:

- persistent impairment in mutual communication and social interaction
- limited and repetitive patterns of behaviour.

In the DSM-V it was proposed to no longer consider Asperger's syndrome as a distinct disorder but to incorporate it within the autistic spectrum category; a new diagnosis of social communication disorder was also introduced to include children with language and social problems. Finally, CDD and Rett's syndrome have been removed from the autism category.

DIAGNOSIS

Autism is a disorder whose aetiology is not yet known, so the only diagnostic criteria are currently based on behavioural indicators defined by the DSM-V drawn up by the American Psychiatric Association, and by the International Classification of Diseases (ICD-10) drawn up by the World Health Organization. In order for a subject to be recognized as autistic he must show, according to the criteria of the DSM-V, 5 characteristics:

1. Persistent deficit in communication and social interaction: communication may include decreased reactivity to speech stimuli, use of poorly diversified words or word combinations, less inclination to make demands and more propensity to simply repeat the words of others. Children aged three to five years have a harder time approaching others spontaneously or in alternating in a discussion. Older children and adults instead show reduced ability to identify and describe their emotions, which results in a reduction in empathy and in the ability to recognize the emotions of the other.
2. Behaviours and/or interests and/or restricted and repetitive activities: this area includes for example, limited interest in surrounding stimuli and repetition of a series of behaviours in the same sequence. Stereotyped behaviour can be varied, from motor

mannerisms, such as clapping hands or flickering of the same, to the vocal ones like the continuous repetition of the same syllable, word or phrase.

3. Symptoms should be present in early childhood.

4. The set of symptoms must compromise the daily functioning.

5. These alterations are not better explained by intellectual disability or late global development [2].

The diagnosis of ASD is exclusively based on behavioural parameters so it is essential to use standardised observation methods and to adopt suitably developed assessment scales for autistic "behaviour". The most widely used diagnostic tools at international level are:

- *Childhood Autism Rating Scale* **(CARS)**: is a scale of evaluation of autistic behaviour, used from the age of 2 years, that allows to explore 15 areas of development: Relating to people; Imitative behaviour; Emotional response; Body use; Object use; Adaptation to change; Visual response; Listening response; Perceptive response; Fear or anxiety; Verbal communication; Non-verbal communication; Activity level; Level and consistency of intellective relations; General impressions. The examiner gives for each area considered a score ranging from 1 to 4 where 1 indicates appropriate behaviour for the age level, while 4 indicates a serious deviation from normal behaviour for the age level. The individual scores awarded are added together into a total score that classifies the child as non-autistic (under 30), mildly or moderately autistic (30-36.5) or severely autistic (over 36.5)[3].

- *Autism Diagnostic Observation Schedule* **(ADOS)**: is a semi-structured and standardized assessment of communication, social interaction, play and imagination, developed to perform diagnostic evaluations in individuals with a possible autism

spectrum disorder. The ADOS comprises several modules that take age and language skills into account. Each module evaluates the 2 important areas for the diagnosis of ASD:

- Social Affection (AS)
- Repetitive Narrow Behaviour (CRR).

The scores assigned to the two areas are added together into an overall total; this is then converted into a score of comparison by means of a conversion table that considers age and language development, specific to the module used. The comparison score ranges from 1 indicating the minimum degree of severity to 10 indicating instead the highest degree [4].

- *Autism Diagnostic Interview-Revised* **(ADI-R):** is a semi-structured and standardized interview with parents, developed to assess the presence of ASD symptoms in early childhood in all three main symptomatologic areas: mutual social interaction, communication, and restricted/repetitive behaviour. Items are classified with scores ranging from 0 (no definitive behaviour of the specified type) to 3 (extreme severity of the specified behaviour). This is usually accompanied by the ADOS [5].

EPIDEMIOLOGY

Since the 1960s, several independent epidemiological studies have been conducted in various geographical regions investigating ASD prevalence rates [6]. These studies have shown a dramatic increase in ASD cases generating claims about an "epidemic" of these disorders. For example, in USA in 2000, the ADDM (Autism and Developmental Disabilities Monitoring) estimated the incidence of ASD to be 1 in 150 children. In 2008, the incidence of ASD increased to 1 in 88 children, in 2012 it increased to 1 in 68 children and in 2014 it increased yet again to 1 in 59 children (Fig.1). The latest estimates on the prevalence of ASD in USA report that in 2016 1 in 54 children has ASD [7].

Fig. 1 Prevalence of ASD in USA

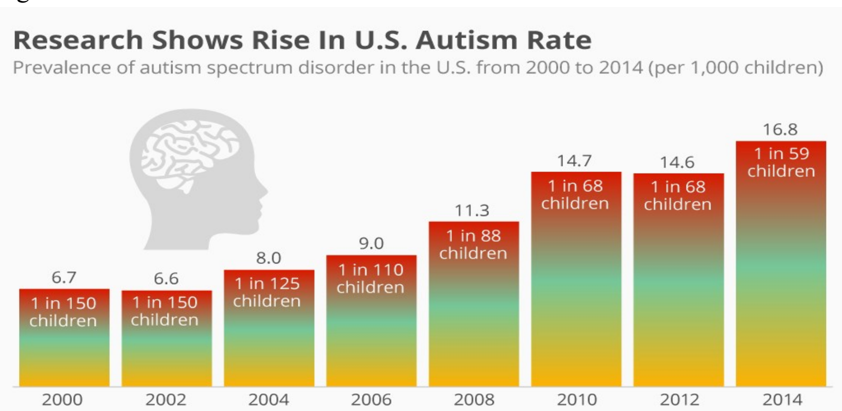


Image taken from: ADDM Network (2018)

In Europe studies carried out before 1999 reported average ASD prevalence rates of about 18,75/10,000; whereas studies carried out after 1999 reported average rates of 61,9/10,000. In 2014 the National Autistic Society reported that in the United Kingdom 1 in 100 children have ASD [8]. Finally, a study conducted in Italy in 2018 showed a prevalence of ASD in children between 7 and 9 years of age of about 1 in 87 [6]. In recent decades the question has been raised whether the steady increase in the prevalence of ASD was real or due to an expansion of screening and diagnostic criteria, a lower age of diagnosis and intervention or increased awareness of these disorders. Among the

most common explanations for the increase in ASD prevalence are the frequent changes over the years in the diagnostic criteria for ASD and the definition of subcategories of autism or autism-related disorders. The formal diagnostic criteria did not emerge until the publication of the third edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-III) in 1980. Diagnoses made prior to this publication were then evaluated using different criteria. These criteria were then modified and expanded in subsequent versions of the DSM (1987, 1994, 2000 (DSM IV-TR)) until its last revision, DSM-5 (2013), in which the definition of autism was modified. Each updated version of the DSM reported several changes concerning factors such as age of onset, inclusion of symptoms and the number of symptoms required for diagnosis [9]. Another potential cause of increased prevalence may be linked to an inaccurate diagnosis. Clinically derived diagnoses are often made following a brief observation using DSM or ICD guidelines, but do not make use of systematic tests or if they are used, they may vary greatly in the type of test used and/or according to the operators. In fact, different professional figures (clinical psychologists, school psychologists, psychiatrists, neurologists) can conduct these assessments and their experience and training with ASD varies greatly. On the contrary, diagnoses made using research criteria would probably be more accurate as homogeneous protocols would be used with a battery of standard tests conducted by professionals with ASD-specific experience and training. Another factor that could influence the ASD prevalence estimates is the fact that these are carried out using data derived from various methods including log reports, telephone interviews and surveys. Social factors, such as education, appear to play a role in the assessment of children with ASD and thus in the overall ASD rates reported. In addition, there has been an improvement in

perinatal and neonatal care in recent decades, resulting in increased survival rates for premature infants. These are more likely to develop neurodevelopmental disorders, including ASD, than the general population and this has led to the hypothesis that both prenatal risk factors and the high rate of neonatal hospitalization may be a possible cause of the increased prevalence of ASD. Therefore, although there is strong evidence that current estimates of ASD prevalence are significantly higher than those reported in previous decades and part of this increase is due to a real increase in prevalence, it is very likely that part of this increase is due to distortions due to the factors mentioned above [10]. Most epidemiological studies have shown a gender disparity in ASD populations with about 4.5:1 times more males affected than females. To date, the mechanisms underlying this sex difference are not known. Determining the processes involved in ASD is very important in order to have a better understanding of the aetiology of such disorders [11]. Several hypotheses have been made about the mechanisms that may be underlying the male preponderance of autism and other neurological developmental disorders. Among them we find:

- **Female Protective Effect (FPE) theory**: suggests that females are biologically protected against ASD compared to males, requiring a higher degree of genetic and environmental risk factors to express the symptoms of ASD. This means that the degree of etiological risk able to cause behavioural expression of ASD in males does not result in the same level of impairment in females. Several studies have shown that women with ASD have more *de novo* mutations than men with the same severity of symptoms [12,13,14]. One explanation for these results could be that more mutations or "hits" are needed to determine ASD in girls than in boys, as if the threshold above which the genetic mutation produces behavioural symptoms was higher in females

than in males [15]. This theory has been supported by animal studies. An example is the study by Grissom and colleagues who showed that mouse females with a deletion in the chromosomal region 16p11.2, which is linked to autism, did not present learning problems which instead were present in males with the same deletion [16]. Further support for the female protective effect has been given by studies that have examined the relatives of subjects diagnosed with ASD. In the 1980s, a study highlighted the fact that females with ASD had on average more relatives with ASD or some language disabilities than males with these disorders [17]. Subsequently, other studies supported these observations. A study showed that siblings of females with ASD are more likely to be affected in turn than siblings of males with such disorders. So, the females would have a protective effect against the ASD but could still transmit the family risk factors to their children [18].

- **Extreme Male Brain theory:** proposes that there are morphological and functional differences between the male and female brains and that the "autistic brain" is a more extreme, or hypermasculine, version of the male brain [19]. It can be considered as an extension of the theory of empathy-systematization (E-S) according to which individuals can be classified on the basis of two dimensions: empathy, i.e. the stimulus to perceive the feelings and thoughts of others and systematization, i.e. the drive to understand rule-based systems [19,21]. Some studies have shown that typical women score significantly higher in empathy measures than typical males who in turn score higher than those with ASD [22]. Regarding systematization, individuals with ASD scored higher than typical males who in turn scored higher than typical females [23,24]. Some results from brain morphology studies support the theory of EMB by reporting gender differences in size or laterality, with ASD individuals showing an exaggerated male

pattern [21]. In addition, MRI studies have shown that females with a typically male brain organization were significantly more likely to receive a diagnosis of ASD [25]. Another recent study reported an accentuation of the "male brain" phenotype in individuals with ASD, in particular, decreased connectivity in the default mode network (DMN), a group of interconnected brain areas whose activity was related to social processing and alterations in ASD [26]. Further studies will be needed to identify the factors that regulate sex-specific neural expressions in ASD and to understand whether these are a cause or effect of ASD.

- Sex Hormone Theory: behavioural and anatomical differences between males and females are based on sex hormones, mainly testosterone and estradiol. These steroids, produced by the gonads, have important effects on development, particularly during two critical periods: during development in utero where they cause the body and nervous system to form in the male or female anatomy; the second critical period occurs during puberty when there is an increase in sex hormones [11].

Fetal testosterone (FT) has been identified as a prime candidate for male-biased risk. Baron-Cohen and colleagues proposed that FT levels can drive cognitive hypermasculinization in ASD [20]. The results of significant positive correlations between FT levels and systematization measures [24] and / or autistic traits [27] and negative correlations with empathy measures [28] and social relationships [29] are consistent with this hypothesis. A recent study also observed a correlation between the increase in FT and the volume of sexually dimorphic brain areas [30]. These results suggest that an increase in FT levels predisposes the brain to a hypermasculin cognitive and neuroanatomical phenotype. Interestingly, the results of several studies have suggested that testosterone can play an important role in the pathophysiology of ASD.

In particular, testosterone levels and its precursors were found significantly elevated in a sample of subjects with ASD [31]. Other subsequent studies have reported an increase in serum androstenedione in adults with ASD compared to healthy controls [32]. These results were examined in a recent study [33], whose results suggested that hyperandrogenism may be a significant risk factor for ASD.

- SEX CHROMOSOME THEORY: it has been hypothesized that having a Y chromosome, in the case of males, is a risk factor for neurodevelopmental disorders, whereas having a second X chromosome would be a protective factor in the case of females. Evidence to support this hypothesis comes from studies on aneuploid individuals showing an increased risk of ASD (XYY, XXYY, XXY) [18,34]. However, these individuals often also show a dysregulation of sex hormones, so it is difficult to interpret these studies and understand whether the effect is due to chromosomal or hormonal alterations. Other evidence may be that most of the genetic mutations associated with ASD susceptibility are autosomal, but some genes reside on the X chromosome (*MECP2*, *NLGN3* and *NLGN4X*) [18,21] and *X-linked* phenotypes in males are often more severe than in females. An example is Rett's syndrome caused by mutations in the *MECP2* gene, found on the X chromosome, characterized by a phenotype like ASD. This syndrome is almost exclusively seen in girls because males, having only one copy of the *MeCP2* gene, are severely affected and usually die before or shortly after birth [35].

- UNDER-DIAGNOSIS OF FEMALES WITH ASD THEORY: proposes that the bias and diagnostic variations in the way ASD is expressed in females means that it is not recognised in females at the same level as it is recognised in males [36]. The diagnostic criteria currently in use have been developed on the basis of predominantly male populations previously identified as autistic [37,38]. It has been suggested that females may be less

likely to meet these criteria resulting in the diagnosis of wider developmental disorders, rather than ASD in particularly [39]. The presentation of ASD in females is qualitatively different from the typical male presentation. The female autistic phenotype has basic characteristics similar to those described in the current diagnostic criteria, i.e. communication and social interaction difficulties, limited interests and repetitive behaviours, but these can be expressed in ways that differ from traditional ASD diagnostic criteria [15,37]. Females may also exhibit additional behaviours or characteristics that are not included in the current ASD diagnostic criteria. An example is the phenomenon of "camouflaging" referring to the use of conscious or unconscious strategies to limit the appearance of autistic characteristics in a social context [15]. Examples of mimicry are: imitating the facial expressions of the person you are talking to or forcing yourself to make eye contact and stop talking only about a particular interest. A similar concept that has recently been proposed is that of compensation or the use of alternative cognitive strategies to cope with socio-cognitive or behavioural difficulties. This compensation may be profound or superficial depending on whether it involves the underlying cognitive processes to achieve the desired result [40].

CAUSES

Although the causes of ASD remain largely unknown today, several studies have shown that genetic, environmental and epigenetic factors play an important role in the aetiology of these disorders.

- GENETICS

In about 5-10% of cases of ASD there are identifiable disorders with a known hereditary pattern, such as Fragile X syndrome, untreated phenylketonuria (PKU), tuberous sclerosis or neurofibromatosis. For the remaining cases of ASD there is no known pattern of specific heredity. Researchers are currently studying the role of genetics in the development of ASD. In order to determine whether a disorder has a genetic basis, it is first necessary to assess whether it occurs in families. Studies report a higher rate of ASD among family members of individuals with ASD than would be expected in the general population. Much of this evidence comes from studies investigating the concordance of ASD between twins. When one twin has ASD and the other twin also meets the criteria for ASD, twins are said to agree with ASD. Studies on monozygotic twins are particularly useful to investigate the genetic basis of a disease. Monozygotic twins share 100% of their genes, while dizygotic twins share about 50% of their genetic heritage. This means that for the development of a disorder to be completely due to genetic factors, monozygotic twins should always be concordant for the disorder [41]. As far as ASD is concerned, studies have reported concordance rates between MZ twins ranging from about 30% to 90%, while the remaining percentage is discordant for ASD [42] . This suggests that several factors are involved in the aetiology of ASD including genetic, environmental and epigenetic factors. However, the concordance rates of MZ twins are higher than those of DZ twins (0-65%) and this

provides evidence of a strong genetic component in the development of ASD [43]. In addition, the reported sibling risk rates for ASD range from about 2.8% to 7.0%, which is much higher than rates found in the general population. This could suggest that the risk of a child suffering from ASD is proportional to the percentage of genome they share with an affected sibling or parent [41,44,45,46]. Early karyotype studies documented chromosomal anomalies and began to shed light on which regions of the genome could be involved in ASD [45,47–52]. Subsequently, the first studies focused on genes mapping suspect chromosome regions that played a critical role in neurodevelopment, such as the homeobox family (*Hox*) or the *Wnt* genes [51,53–55]. Thanks to this approach the involvement of genes such as reelin (*RELN*), aristaless related homeobox (*Arx*), methyl-CpG binding protein 2 (*MeCP2*), neuroligin 3 and neuroligin 4 (*NLGN3*; *NLGN4*), in the aetiology of ASD has been discovered [56–61].

Among the genetic factors involved in the aetiology of ASD there are chromosomal alterations, copy number variations (Copy Number Variations, CNV) and mutations in a single gene. Currently, chromosome aberrations have been reported in about 2-5% of individuals with ASD [62,63]. In particular, the most frequently detected, in 1-3% of children with ASD, is the maternally derived 15q11-q13 duplication [64]. Many of the genes mapping to this chromosomal region, such as *GABRA5* and *GABRB3* (GABA receptors), *UBE3A*, *HERC2* and *SNRPN*, play essential roles in brain function [65–67]. Other chromosomal abnormalities identified in ASD patients include aneuploidies affecting both autosomes (e.g. trisomy 21 or Down syndrome) and sex chromosomes (e.g. Turner or Klinefelter syndrome)[68]. Several studies using microarray technologies, such as Array - Comparative Genomic Hybridization (Array-CGH), have identified about 10% of *de novo* or inherited ASD CNV cases [69]. CNVs are small structural variants (deletions,

duplications, translocations and inversions), sometimes extending over several kilobases (Kb), that result in an alteration in the copy number of a chromosomal region[70]. The CNVs most associated with ASD patients are microdeletions and microduplications in the 16p11.2 region, which were found in about 1% of affected individuals. Most of the 25 genes in this region code for proteins that play essential roles in proper neurodevelopment [71]. Table 1 shows the CNVs most frequently involved in patients with ASD [68]. About 5-10% of ASD cases occur in conjunction with monogenic syndromes. The most common of these is Fragile X syndrome (FXS) diagnosed in about 1.5-3% of individuals with ASD and caused by mutations in the *FMR1* gene that plays an essential role in the regulation of synaptic plasticity [72]. Another frequent ASD-related syndrome is complex tuberous sclerosis (TSC), which occurs in about 1% of patients diagnosed with ASD. The two causative genes, *TSC1* and *TSC2*, act by inhibiting mTOR (mammalian target of rapamycin) and thus allowing a normal synaptic pruning process. About 1% of women with ASD have Rett's syndrome caused by mutations in the *MECP2* gene encoding MECP2 (methyl-CpG binding protein 2), a transcription factor that regulates the expression of many genes in neurons [63]. Other associated monogenic defects include neurofibromatosis, Duchenne muscular dystrophy and Timothy syndrome. In addition, cases of ASD may occur in the presence of metabolic disorders such as phenylketonuria and Smith-Lemli-Opitz syndrome [73].

In recent years the literature has shown a growing interest in identifying genes whose mutation confer only ASD risk (ASD-predominant) and not risk for ID or other NDDs.

In particular, recent studies are attempting to identify ASD-specific genes by comparing the distribution of *de novo* gene mutations between established cohorts of ASD and ID/DD subjects. An example is the study of Satterstrom et al., carried out on a

cohort of 11,986 subjects with ASD, which found the presence of 102 genes implicated in the risk of ASD grouped into three main categories: regulation of gene expression, neuronal communication and cytoskeletal organization. Among these, 53 genes were found to be ASD-specific, while 49 were associated with ASD with ID/DD[74] . Another example is the study by Coe et al, performed on a cohort of ASD individuals (n = 5,624) and a cohort of ID/DD individuals (n = 5,303), which compared the distribution of likely gene mutations between the 2 cohorts examined but reported no evidence of ASD-specificity for any of the 253 genes identified as NDD candidate genes [75]. These results indicate further studies are needed to establish significant specificity for ASD of genes based on large rare-variant data.

Table 1. Recurrent CNVs identified in patients with ASD

Locus	Clinical features associated with CNV
1q21.1 deletion syndrome	Mild to moderate ID, schizophrenia, mild dysmorphic facial features, congenital heart abnormality, microcephaly, cataracts
1q21.1 duplication syndrome	Mild to moderate ID, ADHD, mild dysmorphic features, macrocephaly, hypotonia
2q37 deletion syndrome	ID, dysmorphic facial features, brachydactyly
3q29 deletion syndrome	Mild to moderate ID, schizophrenia, mild dysmorphic facial features
7q11.23 duplication syndrome	ID, schizophrenia, abnormal brain MRI, variable dysmorphic features
15q11q13 duplication syndrome	Mild to severe ID, epilepsy, ataxia, behavioral problems, hypotonia
15q13.3 deletion syndrome	Mild to severe ID, epilepsy, learning difficulties, ADHD, variable dysmorphic features
16p11.2 deletion syndrome	Mild to severe ID, epilepsy, multiple congenital anomalies, variable dysmorphic features, macrocephaly, obesity
16p11.2 duplication syndrome	Mild to moderate ID, ADHD, microcephaly, dysmorphic features
16p12.1 deletion syndrome	Mild to moderate ID, ADHD, congenital heart defects, craniofacial dysmorphology
16p13.1 deletion	ID, schizophrenia, epilepsy, multiple congenital anomalies, dysmorphic features
17p11.2 deletion syndrome	ID, speech delay, hearing loss, sleep abnormalities, hypotonia
17p11.2 duplication syndrome	Mild to severe ID, congenital anomalies, dysmorphic features, hypotonia
17q12 deletion syndrome	Mild to moderate ID, schizophrenia, epilepsy, MODY, dysmorphic facial features
17q21.31 deletion syndrome	Mild to severe ID, epilepsy, structural brain abnormalities, musculoskeletal anomalies, dysmorphic features, hypotonia
17q21.31 duplication syndrome	Mild to moderate ID, microcephaly, hirsutism, facial dysmorphism
22q11.2 deletion syndrome	ID, schizophrenia, learning difficulties, multiple congenital anomalies, congenital heart defect, dysmorphic features
22q11.2 duplication syndrome	ID, schizophrenia, speech impairment, learning difficulties, heart defect, dysmorphic features, microcephaly

ADHD attention deficit hyperactivity disorder, ID - intellectual disability, *MODY* maturity onset diabetes of the young

Image taken from [68]

- ENVIRONMENTAL FACTORS

There is evidence to support a significant contribution of environmental factors in the aetiology of ASD. In the last decade there has been an exponential growth in the number of environmental factors studied in association with ASD. Although studies on twins and the family highlight the importance of a hereditary predisposition to such

disorders, increasing epidemiological data suggest a strong contribution of environmental factors.

The environment can be defined, in the broadest sense, as all non-genetic factors, from viruses to drugs, chemicals or physical agents to social and cultural influences [76]. These factors can be divided into: periconceptional, prenatal and postnatal risk factors.

PERICONCEPTIONAL RISK FACTORS

- Advanced parental age: A recent theory on the aetiology of ASD is that the advanced age of parents at the time of conception may contribute to the risk that offspring develop such disorders. The average age of women facing their first pregnancy has increased in recent decades. Studies show that advanced maternal age is proportionately related to the risk of chromosomal abnormalities in offspring involving genetic disorders such as Down's or Klinefelter's syndrome [77]. In addition, a high maternal age can lead to a higher prevalence of chronic diseases and a less favourable uterine environment, often with a higher risk of obstetric complications that could lead to an increased risk of negative outcomes at birth. In the case of ASD, both the advanced maternal and paternal age at birth (≥ 35 years) have been associated with an increased risk of ASD [78,79]. Studies also show a combined effect of parental age, which is greater when both parents are older [80].

- Use of assisted reproductive technologies: Among the causes involved in the risk of ASD development is the use of Assisted Reproductive Technology (ART). The term ART refers to all those sterility therapy methods that include: IVF (In Vitro Fertilization and Embryo Transfer), GIFT (Gamete Intra Fallopian Transfer - Intratubaric Embryo Transfer), ICSI (IntraCytoplasmatic Sperm Injection), IMSI (IntraCytoplasmatic

Morphologically selected Sperm Injection), TESE (Testicular Sperm Extraction), and MESA (Microsurgical Epididymal Sperm Aspiration) [81]. Evidence suggests that the use of ART increases the risk of congenital malformations, nervous system defects [82], preterm birth, low birth weight, and genetic imprinting disorders [83,84] all of which may explain the development of ASD. In fact, to date, studies on the association between ART use and ASD risk in offspring have provided conflicting results. A recent systematic review reported no significant association between ART use and ASD in offspring [85]. However, a study by Sandin et al. reported that ART procedures for fresh embryo transfer using ICSI (Intracytoplasmic Sperm Injection) may be associated with an increased risk of autism and intellectual disability in offspring compared to fresh embryo transfer procedures not using ICSI [86]. These results are consistent with those present in the study carried out by Kissin et al., which reported that children born from pregnancies in which the ICSI method was used showed a higher risk for the incidence of ASD than the conventional IVF method [87]. Although these studies have proved informative, to date no studies have examined the risk of ASD in offspring in relation to their exposure to ART compared to natural conception.

PRENATAL RISK FACTORS

- Air pollution: In recent years, many studies have found associations between air pollution and the risk of ASD. In particular, a study has shown how air pollutants generated by vehicle traffic (polycyclic aromatic hydrocarbons (PAHs), inhalable particles such as PM 2.5 and PM 10) can be a risk factor for the onset of ASD [88]. Another study reported associations between exposure of children to high levels of carbon monoxide, nitrogen dioxide, ozone or sulphur dioxide and an increased risk of

ASD. In both cases, the risk increased if exposure occurs during the last trimester of pregnancy or the first year of life of the infant [89]. Possible pathophysiological mechanisms concern oxidative stress and/or inflammation. Short- or long-term exposure to high ozone concentrations may induce oxidative stress, leading to lipid peroxidation in the brain and this may cause neuronal damage [90].

- **Heavy metal exposure**: Several studies have shown that long-term exposure to environmental pollution by heavy metals (e.g. arsenic, cadmium, lead) during pregnancy and/or the first year of life could be associated with ASD risk [91,92]. Exposure to lead and mercury has been correlated with neurological developmental disorders. The study by Blanchard et al. for example reported a close correlation between high concentrations of mercury in the environment and the incidence of ASD [93]. Another case-control study reported higher concentrations of heavy metals in the blood, urine, hair, brain and teeth of children with ASD than healthy controls [94].

- **Pesticides**: A small but growing number of literature studies report associations between exposure to pesticides during pregnancy and ASD. A study conducted in a farming community reported a high prevalence of pervasive developmental disorder (PDD) and correlated it with a high concentration of pesticides in the urine of pregnant women [95]. Similarly, in a neuroimaging study Rauh and colleagues showed changes in the volume of speech and social cognition areas of the cerebral cortex in children in whom high levels of the pesticide chlorpyrifos were found in cord blood [96]. The study by Roberts and English showed that the two periods of time most vulnerable to the effects of pesticide exposure are between about 40 days before fertilisation and 160 days after fertilisation and between about 340 and 530 days after fertilisation [97].

- **Medication**: An increasing number of studies have highlighted the potential association between prenatal exposure to selective serotonin reuptake inhibitors (SSRIs) and the onset of ASD, assuming a pathogenic link between serotonin pathway alterations and characteristic neurobiological abnormalities of ASD [98,99]. Another area of interest was the study of antiepileptic drugs (AED). Among these, valproic acid (an antiepileptic drug widely used for the treatment of epileptic seizures and bipolar disorder) showed the strongest association with cognitive impairment, developmental retardation and ASD [100]. Therefore, it is contraindicated in pregnant women or those planning a pregnancy. In addition, other AEDs such as oxcarbazepine and lamotrigine were found to be associated with ASD in offspring [101]. Some studies have also suggested a possible link between the use of antibiotics in the prenatal period or during the first years of life and ASD [102]. Recently a study in mice has reported an association between exposure to low doses of antibiotics in the last trimester of pregnancy and/or early postnatal life in mice and the presence of compromised and aggressive social behaviour in offspring associated with changes in the intestinal microbiome [103]. Indeed, studies have reported that disturbances of the intestinal microbiota during the early stages of life may adversely affect neurodevelopment [104,105]. Very often children with ASD, during the first 3 years of life, are treated with high doses of antibiotics and this can destabilize their intestinal microbiota and thus allow potentially competitive pathogens to exacerbate their symptoms [106]. Studies in germ-free mice have demonstrated an increase in repetitive behaviours similar to those observed in ASD suggesting that the microbiota is a critical factor in the development of social behaviour and the etiology of this pathology [107].

- **Nutritional factors**: Some studies have explored the correlation between maternal nutrition and the risk of ASD in offspring. Maternal nutrition is essential for fetal brain development and nutrient deficiency during pregnancy has been associated with strong increases in risk of schizophrenia, neural tube defects and neurological developmental problems. Nutrient deficiencies are particularly common during pregnancy due to increased metabolic demands and have been shown to influence brain development in terms of structure and function. There is therefore a strong likelihood that maternal nutrition can also influence the risk of ASD [76]. Both maternal obesity and underweight during pregnancy have been associated with an increased risk of ASD [108,109]. Obesity in pregnancy causes activation of the maternal immune system leading to high levels of circulating pro-inflammatory cytokines compared to their normal weight counterparts; it is also related to the presence of chronic inflammation of the uterine environment leading to alterations in fetal cytokine expression, fetal neuronal damage and changes in gene expression in the neonatal brain [110]. Similarly, maternal malnutrition during pregnancy can also cause a physiological stress response leading to neuronal damage through increased release of proinflammatory factors [111]. A diet low in omega-3 and/or high in fat during pregnancy has been associated with the risk of ASD and other neurological development disorders. Fat consumption during pregnancy is strongly associated with the activation of inflammatory cytokines (e.g. interleukins IL-4, and IL-5) that were found to be elevated during gestation of mothers whose offspring later developed ASD. In addition, a fat-rich diet in pregnant women has also been associated with changes in the main neurotransmitter systems, particularly the serotonergic system involved in behavior control [112,113]. A study carried out by Nevison et al. reported an increased risk of ASD in

children whose mothers had followed a diet low in omega-3 fatty acids during pregnancy [114]. Another study has shown that an insufficient intake of omega-3 fatty acids during pregnancy can lead to abnormal neurological and cognitive development in children with concentration deficits, dyslexia, hyperactivity, movement disorders and ASD [115].

- Prenatal infections: Prenatal exposure to infections such as influenza, rubella, measles and bacterial infections may increase the risk for offspring to develop bipolar disorder, schizophrenia and ASD [116]. Recent studies have shown a potential link between the risk of ASD and maternal infection or inflammation during pregnancy. In these studies, the importance of the gestational period during which exposure occurs, the type of infectious agent and the intensity of the maternal immune response have been highlighted. In particular, it appears that viral infections can only be associated with ASD risk if they occur in the first trimester, bacterial infections in the second trimester, while influenza and generic febrile episodes throughout pregnancy but especially in the third trimester [117,118].

- Maternal immune activation: Epidemiological studies over the past decades have led to a growing interest in the relationship between maternal immune activation (MIA) and neurological developmental disorders such as ASD and schizophrenia. A study conducted in New York City on a large cohort of mother-child couples reported that 20% of subjects born to mothers with clinical rubella had been diagnosed with schizophrenia [119], while this disorder has a prevalence of less than 1% in the general population [120]. In addition to rubella, maternal infections with the presence of influenza [119,121], toxoplasma gondii [122,123], cytomegalovirus [124] were also correlated with an increased incidence of neurological development disorders in offspring. This

suggests that immune activation can alter normal brain development. To support this hypothesis, a study conducted in 2010 on about 2 million children born between 1980 and 2005 in Denmark reported a significant association between the risk of ASD and maternal viral infection (during the first trimester of pregnancy) or bacterial infection (during the second trimester of pregnancy) [125]. A more recent study in Northern California reported an association between maternal infection, which requires hospitalization, and an increased risk of ASD in offspring; this study also pointed out that bacterial infection during pregnancy generated the highest risk [126]. Since the placenta is a source of hematopoietic stem cells for the fetus [127], it has been proposed that maternal infection can permanently modify the immune system of the offspring with alterations in the fetal brain immune status [128]. Recent data have identified the presence of fetal brain-specific antibodies in mothers of children with ASD [129–131]. These isolated antibodies produce ASD-like symptoms in offspring of naïve rhesus monkeys when injected into their mothers during pregnancy [132]. These data strengthen the evidence that autoimmune disorders are common in individuals with ASD and their first-degree relatives, suggesting a critical role of immune dysregulation.

- Other maternal factors: Other maternal factors were considered risk factors. Among these, gestational diabetes has been seen to adversely affect fetal growth and increase the rate of complications during pregnancy [133]. In addition, it has a long-term impact on motor development and leads to learning difficulties, attention deficit disorder and hyperactivity [134,135]. These negative effects have been hypothesized to result from increased fetal oxidative stress that can impair brain development or epigenetic changes in the expression of different genes [136,137]. Excessive gestational weight gain (GWG) has also been associated with pregnancy complications and adverse outcomes

for both mother and offspring. This has led to clinical recommendations for weight gain based on maternal pre-pregnancy BMI [138]. To date, few studies have examined this aspect in relation to ASD, with conflicting results**.** An example are the studies of Bilder et al. (2013)[139], Windham et al. (2019)[140], Gardner et al. (2015)[141] that have shown a positive association between GWG and ASD risk.

PERINATAL/EARLY POSTNATAL RISK FACTORS

In recent years it has been suggested that obstetric risk factors could be associated with an increased risk of ASD. These include Caesarean section (CSs), gestational age, low birth weight. Several studies have investigated the possible relationship between CS and/or induced childbirth and the risk of ASD. An example is the study by Glasson et al. (2004) that showed how CS and induced labor might be associated with a higher risk of ASD[142]; these results were also found in other more recent studies[143,144]. The way in which, according to the authors, CS could constitute a risk factor for ASD is through the induction of a dysregulation of the gut microbiota. Today there is general consensus that vaginal delivery is essential in providing the appropriate bacterial substrate for the development of a physiological bowel. In fact, the intestinal microbiota of babies born by vaginal delivery resembles the vaginal microbiota of their mother, which is dominated by Lactobacillus, Prevotella, or Sneathia spp., while the intestinal microbiota of children born by caesarean section is similar to the skin microbiota of their mother, which is dominated by Staphylococcus, Corynebacterium, and Propionibacterium spp. [145]. The possibility that the increased risk of ASD may be linked to imperfect development of intestinal flora is supported by a number of scientific evidence including the fact that the intestinal microbiome of children with

ASD shows a different composition than healthy developing individuals and this could be responsible for the frequent gastrointestinal disorders that characterize patients with such disorders [146,147]. Several studies have reported associations between birth weight (BW), gestational age (GA) and ASD risk. In a case-control study among the Finnish population [148], low birth weight (LBW, BW < 2,500 g) and delivery before 31 weeks of gestation were associated with an increased risk of ASD. The study also reported an increased risk in babies born young for gestational age. Other studies have shown similar associations [149,150]. One hypothesis to explain this association could be the link between the LBW and preterm birth that has been seen in several studies to be associated with ASD [148,150–152].

- **PROTECTIVE FACTORS**

Most of the studies in the literature have focused on the analysis of environmental risk factors for ASD; in recent years a growing body of research has begun to study the role of probable protective factors. Some elements of the mother's diet could play a protective role against ASD. It is known that folic acid intake in the peri-conceptional period can prevent up to 50-70% of neural tube defects and has also been associated with a decrease in childhood behavioral problems, hyperactivity and an improvement in neurological development, attention, verbal and verbal-executive function. Furthermore, several studies have shown that mothers who had taken supplements or folic acid in the prenatal period had a reduced risk of having a child with ASD[153–155]. An average daily intake of folic acid of 400 µg during the periconceptional period and/or early pregnancy has been associated with a 40% decrease in the risk of ASD[156]. This

association is strongest in children and mothers with *MTHFR* variant 677 C > T (cytosine > thymine), which leads to less efficient folate metabolism [154].

Vitamin D supplements during pregnancy may also reduce the risk of developing ASD in offspring [157]. In addition, some studies have provided evidence that breastfeeding (exclusively or accompanied by additional supplements) can protect against the risk of ASD [158]. It has been hypothesized that protection is due to specific components contained in breast milk such as bifidobacteria, lysozyme, glutathione, anti-inflammatory cytokines [158,159]. Support for this hypothesis comes from the literature which suggests that, in relation to healthy controls, children with ASD show lower levels of bifidobacteria and lysozyme in the digestive tract and increased levels of inflammatory cytokines in plasma. Another factor that could play a protective role is melatonin. Melatonin synthesis is often compromised in patients with ASD and their mothers, so its intake during pregnancy could act as a neuroprotective factor, decreasing the risk of neurological developmental disorders, including ASD [160] (Fig.2).

Fig.2 ASD risk factors and protective factors

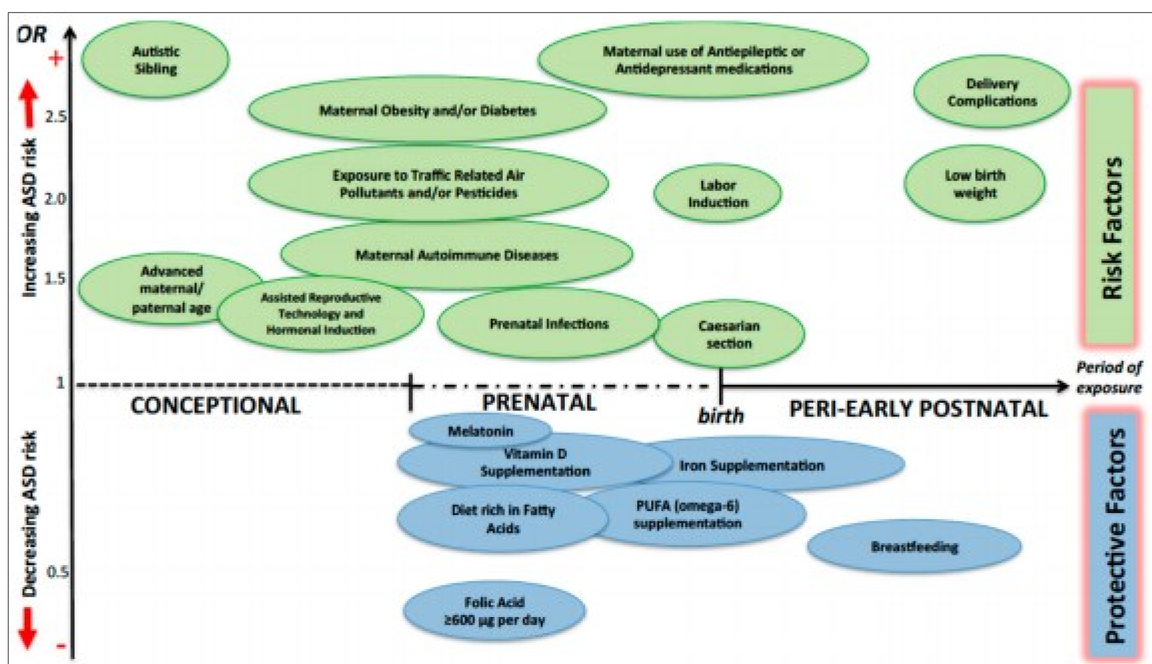


Image taken from [81]

EPIGENETICS

The concept of epigenetics has evolved since the late 1930s when Waddington defined it as the study of causal mechanisms operating during development. Over the years its meaning has changed, up to the present day when the term epigenetics refers to the study of heritable changes in gene expression not resulting from changes in the DNA sequence [161]. These are, therefore, hereditary phenomena in which the phenotype is determined by the superimposition on the genotype of "an imprint" that influences its functional behaviour.

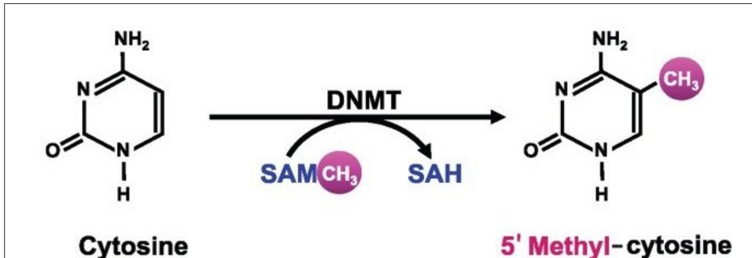
The best-characterized epigenetic mechanisms are DNA methylation, histone modifications and gene silencing by non-coding RNAs. Defects in these mechanisms may be the cause of neurological developmental disorders and are involved in several diseases such as cancer and neurodegenerative diseases [162].

- DNA METHYLATION: consists of the covalent addition of a methyl group at position 5 of the pyrimidine cytosine ring with 5-methylcytosine (5-mC) formation (Fig.3) and is usually associated with gene silencing. In mammals, DNA methylation occurs mainly in the context of cytosine-phosphate-guanine dinucleotides (CpG). While most CpG dinucleotides (about 60% - 80%) are methylated, short DNA regions of about 500 bp, with high concentrations of CpG, known as CpG Islands (CGI), are usually non-methylated even though a small percentage of them become methylated during the normal physiological processes, such as genomic imprinting and inactivation of the X chromosome and when this happens the associated gene is permanently silent and therefore DNA methylation has been considered as a sign of long-term repression. Many of these CGI are located near the promoter of about 40% of the genes of the mammals and when they are methylated, they cause stable transcriptional gene

silencing. These CGI are one of the initiation sites where the transcription factors bind to the DNA allowing transcription of the corresponding gene. When the promoter contains 5-mC in their CGI, transcription factors are not able to bind to the DNA, which causes gene silencing by transcription inhibition. On the contrary, the function of DNA methylation outside the gene promoter region is not yet fully clarified. It is thought that methylation in intragenic regions may be involved in alternative splicing; or that methylation of transposable elements may prevent translocation phenomena and therefore may be associated with maintaining chromosomal stability [163]. Cytosine methylation is performed by enzymes called DNA-methyltransferases (DNMTs) that are part of the group of epigenetic "writers", i.e. enzymes that catalyze the addition to DNA or histones of specific molecules such as methyl and acetyl groups. These enzymes are grouped in a family composed by 5 types of DNMT proteins: DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L. The function of DNMTs in DNA methylation can be divided into methylation of maintenance and methylation *de novo*. DNMT1 is involved in the methylation of maintenance, which refers to the process of copying DNA methylation profiles on new filaments during DNA replication. DNMT3A and DNMT3B are *de novo* methyltransferases that establish DNA methylation patterns in early development. DNMT3L has no catalytic activity but can help *de novo* methyltransferases by improving their ability to bind to the DNA and stimulate their activity. DNMTs take the methyl group from S-adenosyl methionine (SAM), producing S-adenosyl homocysteine (SAH). SAH is hydrolysed to homocysteine (Hcy) and adenosine. DNA demethylation in the mammalian genome occurs through two types of processes: a passive methyl group removal process that occurs during subsequent DNA replication cycles due to the lack of DNMT1, and an active enzyme-mediated

removal carried out by the TET (ten-eleven translocation) family of proteins which progressively oxidize from 5-mC to 5-hydroxymethylcytosine (5-hmC), 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC) [164].

Fig.3 Schematic representation of DNA methylation



- HISTONE MODIFICATIONS:

In human cell nuclei, the genomic DNA is packed into nucleosomes, the fundamental units of chromatin. Each nucleosome consists of an octamer made up of two copies of four core histones (H3, H4, H2A, H2B), around which 150 base pairs of DNA bases are wrapped. The histones of the nucleosome's central body have a tail of N-terminal amino acids that projects outwards. These tails undergo various post-translational modifications such as acetylation, methylation and phosphorylation, which are responsible for conformational changes in chromatin leading to repression or activation of transcription [165]. Acetylation is mediated by the histone acetyltransferase (HAT) enzymes that catalyse the transfer of an acetyl group to lysine residues on histone tails; deacetylation, i.e. the removal of the acetyl group is carried out by the enzymes histone deacetylase (HDAC). Acetylation of lysine residues on the tails of the histones neutralizes their positive charge and decreases their affinity for DNA, leading to the relaxation of the chromatin structure, called eucromatin, with transcriptional promotion. Deacetylation of the histonic tails, on the other hand, promote a more

chromatin condensate, called heterochromatin, which involves decreasing or silencing of the gene transcription [166].

- Non-coding RNAs (ncRNAs): are a large class of RNA that are not translated into proteins. Their discovery was a turning point in science as it gave birth to a new vision of the "central dogma" of biology (DNA→RNA→Protein) and to the theory of "junk RNA", which referred to all those RNAs that were not translated - about 70% of the genome is transcribed, but only about 2% is then translated - and that therefore, it was believed, had no function. Today it has been widely demonstrated that these molecules instead play a very important role in the regulation of the translation and transcription of encoding and non-coding genes. According to their size they can be classified into “small ncRNAs” (<200-300 bp), which include microRNAs (miRNAs), short interfering RNAs (siRNAs), endo-siRNAs, PIWI-interacting RNAs (piRNAs) and “large ncRNAs” (lncRNAs >200-300 bp) (Maia 2014). The miRNAs are short ncRNAs of about 20 - 24 nucleotides that act as negative, post-transcriptional regulators of gene expression by binding to the 3' untranslated region of the target mRNA with a consequent translation repression or degradation of the mRNA itself. Their biogenesis takes place in several steps: the first phase takes place in the nucleus where the miRNA is transcribed with the production of a first precursor called pri-miRNA. The second phase involves the nuclear cleavage of the pre-miRNA, carried out by Drosha RNase III endonuclease with the formation of the pre-miRNA, about 70 nucleotides long, that is transported into the cytoplasm where it is further processed by the complex Dicer in mature miRNAs and become part of the RNA induced silencing complex (RISC). miRNAs play a very important role during development and throughout life. It is therefore not surprising that a significant number of miRNAs have been found to be deregulated in a

variety of conditions including psychiatric, neurodevelopmental, neurodegenerative, and cardiovascular diseases, as well as various cancers [167].

EPIGENETICS AND ASD

METHYLATION ANALYSIS OF ASD CANDIDATE GENES

Large-scale genomic studies have shown the presence of single nucleotide and copy number variants in about 100 genes and genetic loci highly penetrating in ASD [148,168,169]. Most ASD-related genes can be grouped into two distinct functional classes: genes encoding proteins involved in the development and function of synapses and genes encoding enzymes or proteins important for the regulation of chromatin and epigenetic mechanisms [168]. The discovery of genes related to synaptic function was predictable given the neurological nature of these disorders; on the other hand, the discovery of the large number of enzyme and protein encoding genes involved in chromatin modification and DNA methylation related to ASD was unexpected. This evidence suggested how epigenetic dysregulation has a key role in the neurological development and pathogenesis of neurodevelopmental disorders including ASD [170] (Table 2).

- **METHYL-CpG BINDING PROTEIN 2 (*MECP2*)**

One of the best studied epigenetic factors associated with ASD is the methyl-CpG binding protein 2 (*MECP2*), an important epigenetic regulator of brain development, very rich in the central nervous system, particularly in GABAergic interneurons. Abnormal levels of *MECP2*, including overexpression by gene duplication or loss of expression by mutation, leading to *MECP2* duplication syndrome or Rett syndrome

respectively, are known to be associated with behavioural disorders like those found in ASD [171]. One study reported a decrease in *MECP2* expression in the frontal cortex of ASD patients, associated with abnormal methylation on the promoter of this gene [172]. In another study with 14 patients with non-syndromic autism, the MeCP2 protein was significantly reduced in the frontal cortex of 11 autistic subjects compared to control subjects. This decrease in protein expression was correlated with higher levels of methylation in the *MECP2* gene promoter in cases compared to patients. These results suggest a direct link between gene expression regulated by DNA methylation and ASD symptoms [173].

- **OXYTOCIN RECEPTOR (*OXTR*)**

Another gene involved in ASD aetiology is the oxytocin receptor gene *OXTR*. Oxytocin is known for its role in various aspects of social behaviour.

Methylation analyses performed on peripheral blood samples from 20 individuals with ASD (10 males and 10 females) and 20 healthy controls (10 males and 10 females) showed a significant increase in *OXTR* methylation in individuals with ASD compared to controls; in the same study a similar methylation pattern was reported by analyzing methylation levels in DNA extracted from *post-mortem* brain tissue samples (temporal cortex) of 8 ASD individuals and 8 healthy controls. In males (6 out of the 8 ASD individuals included in the study), the increase in methylation levels corresponded to a 20% decrease in *OXTR* mRNA expression levels [174]. These results suggest a relationship between increased methylation and decreased *OXTR* gene expression.

In another study carried out by Elagoz Yuksel and collaborators on peripheral blood samples from 27 children with ASD (4 females and 23 males) and 39 healthy controls (6

females and 33 males), the authors analyzed the levels of methylation of four consecutive regions (MT1, MT2, MT3 and MT4) in *OXTR* gene promoter finding a lower frequency of methylation only in MT1 and MT3 regions in subjects with ASD [175] . Finally, a recent study carried out on DNA extracted from saliva samples from patients with ASD and healthy controls studied the relationship between *OXTR* methylation (Introne 1 and exon 1 of the MT2 region in gene promoter), the diagnosis of ASD and the function of brain areas involved in social cognition and data on the social aspect. The results showed increased levels of methylation in intron I of the MT2 region of the *OXTR* gene promoter in ASD; this hypermethylation was correlated with clinical symptoms and hypoconnectivity of the brain areas considered. In addition, the study showed a positive correlation between MT2 exon I methylation, social responses and brain hyperconnectivity. No gender differences were found [176].

- **Reelin (*RELN*)**

Reelin is a glycoprotein that plays an important role both during development, as it regulates neuronal migration and brain lamination, and during adult life being involved in maintaining synaptic function. Disorders such as autism, schizophrenia, bipolar disorder, and depression have in common the presence of an abnormal expression of the reelin protein in the brain. It has been hypothesized that such altered expression may compromise neuronal connectivity and synaptic plasticity, thus leading to cognitive deficits found in such disorders. The mechanisms that cause this alteration are currently unknown. The most widely accepted hypotheses include mutations of the Reelin gene (*RELN*), hypermethylation of the promoter of this gene, mRNA silencing by miRNA [177]. *Post-mortem* brain tissue studies have reported a reduction in both brain

and cerebellar expression levels of this gene in subjects with ASD compared to healthy subjects [178]. Expression of the gene is probably regulated by epigenetic factors: for example, the study conducted by Lintas and collaborators in 2016 revealed a difference in methylation pattern between ASD patients and healthy controls. In addition, a significant reduction in *RELN* mRNA levels was found in ASD cases compared to controls [179].

- **Brain-derived neurotrophic factor (*BDNF*)**

The brain-derived neurotrophic factor (*BDNF*) is part of the neurotrophin group. During development it plays a key role in survival, migration, neuronal phenotypic differentiation as well as in the growth of axons and dendrites and the formation of synapses. In adult life its main function is to regulate synaptic plasticity and it is involved in learning processes, memory and behaviour. *BDNF* is widely expressed throughout the mammalian brain, including the cerebral cortex, hippocampus, basal forebrain, striatum, hypothalamus, brainstem, limbic structures and cerebellum. This makes it a key factor in learning and memory, reward processes, cognitive functions and circuit formation. The role played by the BDNF in promoting neuronal survival and growth lays the foundation for its possible role in the pathogenesis of neurodegenerative diseases [180].

Several studies have found an increase in systemic BDNF levels in ASD patients, compared to controls [181–186]. A potential involvement of *BDNF* gene in ASD also derives from studies of its expression. An example is a study conducted by Taurines and collaborators that reported a reduction in mRNA levels, extracted from whole blood, in patients with ASD compared to healthy controls combined by age and gender [187]. A

possible mechanism that could explain this reduction in expression is an alteration in methylation levels of the *BDNF* gene. In this regard, a study carried out by Wang and colleagues on DNA extracted from peripheral blood cells from 5 children diagnosed with ASD DSM-IV and 5 healthy controls reported the presence of over 200 genes significantly methylated between cases and controls. In particular, compared to the control group, 19 genes were found to be hypermethylated in the affected group, including the promoter of the *BDNF* gene[188].

- **SEROTONIN RECEPTOR (*5-HTR1A*)**

Serotonin (5-HT) is a biologically active molecule with many physiological functions in the mammalian body, present both in the brain and in peripheral tissues. The serotonergic system plays an important role in brain development, which means that aberrations in this system can alter key processes in the developing brain and may be implicated in the pathophysiology of developmental disorders including ASD. Confirming this, several studies have found high levels of 5-HT in the blood (hyperserotoninemia) of about one third of individuals with ASD [189,190]. It has also been seen that variations in *5-HTR1A (*5-HT1A receptor) gene expression may have a potential role in the development of ASD. In particular, the study carried out by Yahya and collaborators in 2019 reports a reduction in expression levels of this gene in the group of ASD patients (n=30 including 18 males and 12 females) compared to healthy controls (n=20) [191]. These evidences may suggest a dysfunction of the 5-HT system in autism. A possible mechanism that could explain this reduced expression is alterations in methylation levels of this gene, but there are currently no studies in the literature correlating the expression of that gene with its methylation levels in ASD patients [192].

- **B-CELL LYMPHOMA 2 (*BCL-2*)**

BCL-2 (B-cell lymphoma 2) is an apoptosis regulatory protein [193] and its gene has been linked to ASD [194]. Studies have reported a decrease in *BCL-2* expression in the cerebellum and frontal cortex of subjects with ASD [195,196]. In addition, changes in the methylation profile of DNA extracted from lymphoblastoid cells of ASD patients have recently been reported showing a decrease in *BCL-2* gene expression [197].

- **ENGRAILED-2 (*EN2*)**

Engrailed-2 (EN2) is part of a class of transcription factors that are homologous in their DNA binding domain called homeobox. The homeobox transcription factors regulate gene expression by binding to AT-rich DNA elements and play a central role in coordinating development. Engrailed-2 (EN-2) is considered an ASD susceptibility gene based on neuroanatomical similarities between ASD and abnormalities of cerebellar development in rodent models. In mice, En2 is highly expressed in Purkinje cells during fetal and early postnatal development, where it acts primarily as a transcriptional repressor until it is downregulated during the perinatal period. It is also expressed in neurotransmitter systems that have been implicated in autism such as serotonin. En-2 $^{-/-}$ knockout mice show a reduction in cerebellum size and cell count, with a reduction of about 30-40% of major brain cell types including Purkinje cells [198]. Although human studies are limited, expression analysis in fetuses at 40 weeks of gestation has indicated widespread gene expression of EN2 in brain regions including cerebellar cortex and deep nuclei. Some studies report reductions in the number of Purkinje cells in the brain of subjects with ASD that appear to occur in the late prenatal period coinciding with downregulation of EN2 [199]. James et al. conducted a study on post-

mortem cerebellar samples of 13 individuals with ASD and healthy controls and found higher levels of *EN2* promoter methylation in affected individuals than in controls. They also found a significant increase in *EN2* gene expression and protein levels. These results may suggest that normal *EN2* downregulation, which signals the maturation of Purkinje cells during late prenatal and early-postnatal development, may not occur in some individuals with autism and that the presence of postnatal *EN2* overexpression may contribute to the cerebellar abnormalities found in ASD [200].

Table 2. Epigenetic studies of ASD

Gene analyzed	Type of sample analyzed and method	Study population	Results	References
***MECP2* Promoter**	*Post-mortem* brain tissue (Frontal cortex) (Immuno-blotting, sequencing)	- 14 ASD patients and healthy controls	- Reduced MECP2 protein expression levels and increased *MECP2* gene methylation levels in subjects with ASD	172
***OXTR* Promoter**	Peripheral blood samples (Sequencing)	- 20 ASD individuals (10 M and 10 F) - 20 healthy controls (10 M and 10 F)	- Increase in *OXTR* methylation in individuals with ASD compared to controls	174
	Post-mortem brain tissue samples	- 8 ASD individuals (6 M and 2 F) - 8 healthy controls	- Increase in *OXTR* methylation in ASD patients. In males this increase corresponded to a 20% decrease in *OXTR* mRNA expression levels	
***OXTR* Promoter**	Peripheral blood samples MSRE-PCR	- 27 ASD subjects (4 F and 23 M) age ≅ 39 months - 39 healthy control (6 F and 33 M) age ≅ 43 months	- Lower frequency of methylation of the *OXTR* gene in cases compared to controls - No difference in terms of gender	175
***OXTR* Promoter**	Saliva samples (Array)	- 35 ASD subjects - 74 healthy controls (17 M and 57 F) age ≅ 28 years	- Increased methylation levels of *OXTR* gene promoter in ASD and correlation with clinical symptoms - Positive correlation between methylation and social responses - No gender differences	176
***RELN* Promoter**	*Post-mortem* brain tissue (Sequencing and Real-Time PCR)	- 6 ASD individuals (5 M and 1 F) - 6 healthy controls (5 M and 1 F)	- Different methylation pattern between ASD and controls - reduction in *RELN* mRNA levels in ASD	179
***BDNF* Promoter**	Peripheral blood samples (Array)	- 5 ASD subjects - 5 healthy controls	- 19 genes hypermethylated in ASD group, including *BDNF* gene	188
5-HTR1A	RNA isolated from whole blood (qRT-PCR)	- 30 ASD subjects (18 M and 12 F) - 20 healthy controls (10 M and 10 F) Median age ≅ 9 years	- Reduced expression levels in ASD	191
BCL-2	Lymphoblastoid cell lines derived from B-cell (LCL) (Microarray)	3 pairs of monozygotic twins that differed in the severity of ASD and their non-autistic siblings	- Many candidate genes differentially methylated between discordant MZ twins as well as between both twins and non-autistic siblings	197
EN2	*Post-mortem* cerebellar samples (McrBC-PCR restriction assay)	- 13 individuals (4 F and 9 M) with ASD - 13 healthy controls	- Higher levels of *EN2* promoter methylation in affected individuals than in controls	200

EPIGENOME-WIDE ANALYSIS OF ASD

In recent years progress in methylation arrays has made it possible to study methylation patterns throughout the genome. Such studies are commonly defined as epigenome wide association studies (EWAS) and are usually conducted following two different approaches: those based on sequencing after bisulphite treatment and those based on affinity for methylcytosine [201]. Very important in methylation studies is the type of tissue used, the methylation patterns in fact vary a lot from tissue to tissue. As for ASD, being a pathology with involvement of the nervous system, investigating methylation models in brain tissue can provide more accurate information on the biological mechanisms underlying the pathology.

- GENOME-WIDE ANALYSES OF DNA METHYLATION IN THE BRAIN IN ASD

Some studies have investigated methylation at the genome wide level in *post-mortem* brain tissue samples of autistic and non-autistic subjects. Ginsberg and colleagues compared differentially methylated gene networks in DNA samples isolated *post-mortem* from the cerebellar and occipital cortex of nine cases with ASD and nine healthy controls. No changes in DNA methylation were identified in autistic brain regions [202]. In another study, methylation profiles were analyzed in brain tissue samples (prefrontal cortex, temporal and cerebellum) of subjects with ASD and healthy controls. The results showed the presence of four significant autism-associated differentially methylated regions (DMRs) [1]. Nardone and colleagues identified several differentially methylated CpG sites in DNA extracted from *post-mortem* brain tissue (prefrontal cortex, anterior cingulate gyrus) of subjects with ASD and healthy controls [203]. In order to understand how methylation affected gene expression in ASD brains, they compared the DMR found with transcriptomic data from ASD *post-mortem* brain

tissue published by Voineagu and colleagues. They noted that the hypomethylated regions were overlapping with genes showing greater expression [204].

- **GENOME-WIDE ANALYSIS OF DNA METHYLATION IN PERIPHERAL TISSUES IN ASD**

Parallel to *post-mortem* brain tissue studies, some studies have investigated methylation in peripheral tissues in ASD. The first study of genomic DNA methylation in ASD on peripheral tissue analyzed DNA extracted from lymphoblastoid cell lines derived from three pairs of monozygotic twins that were discordant for ASD and siblings from two of these pairs. This study revealed many differently methylated candidate genes between both the discordant monozygotic twins and between these and the unaffected siblings [197]. The genes reported included *BCL-2* and *RORA* (retinoic acid-related orphan receptor alpha). The authors also showed a decrease in the expression of both genes in the cerebellum and frontal cortex of subjects with ASD through immunohistochemistry analysis. Wong and colleagues examined methylation levels in blood samples from 50 pairs of monozygotic twins who were discordant for ASD and reported different methylated genes between the twin diagnosed with ASD and the non-asymptomatic twin, including genes previously involved in the pathogenesis of ASD including *AFF2*, *AUTS2*, *GABRB3*, *NLGN3*, *NRXN1*, *SLC6A4* and *UBE3A*. The authors also found significant differences in DNA methylation levels between pairs of twins with ASD that differ for autistic traits (social interaction, communication, stereotypical behaviour). Finally, significant correlations were found between DNA methylation at different CpG sites and symptomatological severity [205].

AIM OF THE STUDY

The aims of this thesis were to evaluate, in a population of 42 girls affected by ASD, the correlation between methylation levels of candidate genes previously associated with this disorder, maternal risk factors and the symptomatological severity. To this end, the experimental protocols for the analysis of genes (*MECP2*, *OXTR*, *BDNF*, *5-HTR1A*, *RELN*, *BCL-2*, *EN2)* were developed using the Methylation Sensitive-High Resolution Melting technique. The anamnestic data were collected through the administration of a questionnaire to mothers on their lifestyle before and during pregnancy, while the symptomatological severity of the girls was evaluated using the "gold standard" ADOS-2 psychodiagnostic tool. We also recruited 25 ASD affected boys, as comparison population, in order to assess the presence of any differences between the two genders.

MATERIALS AND METHODS

- STUDY POPULATION

In this study, a total of 67 children (42 girls and 25 boys) aged 8 years or less with a diagnosis of ASD were analyzed (Table 3). The subjects were recruited at the UO3 department of the IRCCS Fondazione Stella Maris (Pisa), and underwent blood sampling and psychodiagnostic evaluation (ADOS-2) by the multidisciplinary team led by Prof. F. Muratori (Table 4). At the institute, the mothers of the recruited ASD children were given a survey aimed at investigating their lifestyle both in the period near conception and during pregnancy (Table 5).

Table 3. Study population

	GIRLS	BOYS	GIRLS+BOYS	MOTHERS
NUMBER OF SAMPLES	42	25	67	62*
AGE (MEAN ± SD)	4.86 ± 2.01	3.75 ± 1.26	4.35 ± 1.79	33 ± 5.5

* The different sample size is due to the presence of 3 pairs of siblings in the study population.

Table 4. Symptomatological severity

	SYMPTOMATOLOGICAL SEVERITY*		
	LOW	MODERATE	HIGH
GIRLS	4	24	11
BOYS	5	6	9
GIRLS+BOYS	9	30	20

*Symptomatological severity was classified in low, moderate and high using the comparison score obtained through ADOS-2 psychodiagnostic tool conversion table (Fig.5).

Table 5. Survey

INTERVIEW ON PRE- AND PERINATAL FACTORS POTENTIALLY INVOLVED IN THE PATHOGENESIS OF ASD SPECTRUM DISORDER

Name and surname of the child		Date of Birth	
Name and surname of the mother		Date of Birth	
Name and surname of the father		Date of Birth	

Pregnancy order	

DURING PREGNANCY:

Where did you live during your child's pregnancy? In an urban or rural setting?	

Did you take alcohol or drugs during pregnancy? How many times a week?	
Did you smoke while pregnant? How many cigarettes per day?	

Did you take any medications during your pregnancy? Which ones? In which trimester? What dosage?	
In particular, have you ever taken valproic acid during pregnancy?	

Have you taken folate therapy, or vitamin supplements during pregnancy? (Specify)	
Have you had any contact with paints, solvents, detergents? (At the time of conception and during pregnancy)	

Were you used to drink tap or bottled water during the same period?
What work were you doing at the time of conception and pregnancy?

Did you live or work in a building with linoleum floors? (At the time of conception and during pregnancy)	

Have you had any episodes of fever or flu during pregnancy? In which trimester? What type of therapy did you take?

Did you suffer from any of the conditions listed below during your pregnancy?	
Oncological pathologies? Which ones?	
Cardiovascular disease? Which ones?	
Neurological pathologies? Which ones?	
Psychiatric disorders? Which ones?	
Autoimmune diseases? Which ones?	
Diabetes	

Has your pregnancy been complicated by any issues? Which one? (ex. infections, diabetes, gestation)

Did any stressful events occur during pregnancy?	
Death of a relative	
Death of a friend	
Job loss	
Loss of partner's job	
Eviction or loss of home	
Abuse or violence	
Moving or change of residence	
Divorce or separation from your partner	
Earthquakes, floods, hurricanes or other natural disasters	
Legal problems	
Problems with superiors at work	
Decline in household income	
Household debt	
Other	

RELATIVE TO BIRTH:

At what gestational age was the baby born?	

How did the birth occur?	
Natural	
Natural induced	
Dystocic (ex. use of forceps or obstetric sucker	
Scheduled cesarean section	
Emergency cesarean section	

What was the child's APGAR index? (at 1 and 5 minutes)	

How much did the baby weigh at birth?	

How large was the child's head circumference?	

Were there any complications in the days following the birth of the baby/children? (ex. Neonatal seizures, jaundice, cyanosis, etc.?

How much did you weigh at the birth of your child? How tall is she? How much has her weight increased during pregnancy?	

AFTER BIRTH:

How was the baby breastfed? For how long?

Have you suffered from post-partum depression?	

Did your child have prolonged antibiotic therapy during very early childhood?

- **ANALYSIS OF DNA SAMPLES**

The genomic DNA was extracted from whole blood using the "QIAamp® DNA Mini and Blood Mini Kit" (QIAGEN, Milan, Italy) following the instructions provided in the manual and was subsequently quantified with the NanoDrop 2000C (Thermo Scientific) instrument. DNA samples were then treated with sodium bisulphite using the "EpiTect® Bisulfite Kit" (QIAGEN, Milan, Italy), using 200 ng of DNA. All experiments in the study were performed with a C1000™ Thermal Cycler combined with Bio-Rad's CFX 96™ Real-time System. The results obtained were acquired from the thermal cycler itself and analyzed using Bio-Rad's "Precision melt analysis™ software".

- **GENE-SPECIFIC METHYLATION ANALYSIS**

The primers used for the amplification of *MECP2*, *OXTR*, *BDNF*, *5-HTR1A*, *RELN*, *BCL-2* and *EN-2* genes have been designed using the MethPrimer software following the guidelines of Wojdacz and collaborators [206]. Table 6 shows the main characteristics of the primers used. Methylation analysis by MS-HRM were performed on a final volume of 10 µL for each sample, containing 5 µL lx of EpiTect HRM Master Mix (QIAGEN, Milan, Italy), 3 µL of water, 0.5 µL (10 pmol) of forward primer, 0.5 µL of reverse primer (10 pmol) and 1 µL of genomic or standard DNA containing about 10 ng of DNA. The Master Mix consists of Taq polymerase, MgCl2, dNTPs and EvaGreen as DNA interlayer which when excited at a wavelength of 470 nm emits fluorescence at 510 nm. Standard control DNA with 100% and 0% methylation level "EpiTec methylated and unmethylated human control DNA, bisulfite converted" (QIAGEN, Milan, Italy) are used. Starting from these two standards and making dilutions, standards with known intermediate methylation percentages of 12.5%, 25%, 50%, 75% were obtained. The

MS-HRM analyses were performed using a CFX96^{TM} Real-Time PCR detection system (Bio-Rad, Milan) with the following protocol:

Denaturation of 12 min at 95°C in order to activate the polymerase

- 30 s at 95°C
- 45 s at the ideal annealing temperature for each prime
- 45 s at 72°C

} for 50 cycles

Immediately after PCR the samples were subjected to:

- denaturation of 10 s at 95°C
- renaturation of 1 min at 50°C
- temperature increase for melting analysis of 0.2°C every 15 seconds in a temperature range between:

55°C - 65°C *BDNF*

51°C - 61°C 5-*HTR1A*

52°C – 62°C *MECP2*

50°C – 60°C *OXTR*

52°C - 62°C *RELN*

47°C – 57°C *EN2*

For *BCL-2* gene the temperature increase for melting analysis was 0.1°C every 30 seconds in a temperature range between 54°C – 64°C.

A different ideal annealing temperature was found for each primer pair used:

- 58°C *BDNF*
- 52°C *5-HTR1A*
- 62°C *MECP2*

- 53°C *OXTR*
- 56°C *RELN*
- 55,5°C *EN2*
- 61°C *BCL-2*

The data acquired by the thermal cycler were analyzed using Bio-Rad's "Precision melt analysis™ software" which reports the results in the form of a report. In order to obtain an accurate methylation value, we used the "polyfit" function within the MatLab program, which provides an interpolation curve [207]. For the construction of this curve we used the average of the normalized RFU (Relative Fluorescence Unit) values of the DNA standards (0%, 12.5%, 25%, 50%, 75%, 100%) provided to us by "Precision melt analysis™ software" (BioRad, Milan). We then proceed with the insertion of the RFU value of each sample in the function, in order to obtain a precise value of methylation.

Table 6. Main characteristics of the primers used to study gene methylation levels

GENE	PRIMER SEQUENCE	Ta	AMPLICON LENGTH	CpG SITES
BDNF	F: 5'–GGGTTGTTAATTTATATTTGGGAAGT-3' R: 5'-AACCACTAATTACCCACAAAAACC-3'	58 °C	119 bp	4
5-HTR1A	F: 5'-TGTTTGTTAGTGGGGAGATTTTAGT-3' R: 5'-CAAAAACCCAAACAAAAAATTCTTA-3'	52 °C	251 bp	15
MECP2	F: 5'-AATTAAGGTTTTTTAGTTGGGGTAA-3' R: 5'-TTAACCCTCTATCCACAAATACACC-3'	62 °C	145 bp	5
OXTR	F: 5'-AATTATTGTAAAATAAATTTATTTGTTAAG-3' R: 5'-AACTAAAATCTCTCACTAAAACCTC-3'	53 °C	274 bp	26
RELN	F: 5'-TTGAAGAGTTTAGAAGTAATGAATAATAGA-3' R: 5'-ACCTCATCTATAAAAAATTTTAAAATAAAA-3'	56 °C	192 bp	7
EN2	F: 5'-TATTGAGGTTTTGTTTGTAAAATTAG-3' R: 5'-AATAAAAAATAAAACACTAACCC-3'	55.5 °C	219 bp	17
BCL2	F: 5'-GTGGTTTAGAGGAGGGTTTTTTT-3' R: 5'-AATACCTATCCTCTTACTTCATTCTCTACA-3'	61 °C	110 bp	5

- **DIAGNOSTIC TOOLS**

The diagnostic tool used to assess the severity of symptoms of the children recruited for the present study is the ADOS-2 which allows the standardized observation of behavior related to the areas of language, communication, social interaction and repetitive behaviors. The ADOS-2 is organized according to a modular system (5 modules: Toddler, Module 1, 2, 3 and 4), which takes into account age and language skills (Fig.4).

Each module assessed the two key areas for diagnosis (DMS-5 criteria):

- Social Affect (SA)
- Restricted and Repetitive Behavior (RRB) (Fig.5)

The scores assigned to the two areas are added together in an overall total; this is then converted into a comparison score by means of a conversion table that takes into account age and language development, specific to the module used. The comparison score ranges from 1, which indicates the minimum degree of severity, to 10 that indicates the highest degree (Fig.5).

Fig.4 Guidelines for selecting ADOS-2 modules

ADOS-2 module	Chronological age range	Expressive language level
Toddler	12–30 months	No speech, single words
1	31 months and older	No speech, single words
2	Any age	Phrase speech
3	Child, young adolescent)	Fluent speech
4	Older adolescent, adult	Fluent speech

Fig.5 ADOS-2 module and comparison score

ADOS-2 Module 1 Algorithms

Child ID: ______________________ Examiner: ______________________

Gender: Female ☐ Male ☐ Date of Birth: ____________ Date of Evaluation: ____________ Chronological Age: ____________

CONVERTING ITEM CODES TO ALGORITHM SCORES

- **Convert** assigned ratings of **3** to algorithm scores of **2**.
- **Convert** assigned ratings other than 0, 1, 2, or 3 (i.e., 7, 8, and 9) to algorithm scores of **0**.
- **Transfer** assigned ratings of **0**, **1**, and **2** directly to the algorithm form (do not convert).

	CHOOSING THE CORRECT ALGORITHM COLUMN	
	FEW TO NO WORDS Assigned rating of 3 or 4 on Item A1, "Overall Level of Non-Echoed Spoken Language."	SOME WORDS Assigned rating of 0, 1, or 2 on Item A1, "Overall Level of Non-Echoed Spoken Language."
Social Affect (SA)		
Communication		
Frequency of Spontaneous Vocalization Directed to Others (A-2)		
Pointing (A-7)		
Gestures (A-8)		
Reciprocal Social Interaction		
Unusual Eye Contact (B-1)		
Facial Expressions Directed to Others (B-3)		
Integration of Gaze and Other Behaviors During Social Overtures (B-4)		
Shared Enjoyment in Interaction (B-5)		
Showing (B-9)		
Spontaneous Initiation of Joint Attention (B-10)		
Response to Joint Attention (B-11)		
Quality of Social Overtures (B-12)		
SA TOTAL		
Restricted and Repetitive Behavior (RRB)		
Restricted and Repetitive Behaviors		
Intonation of Vocalizations or Verbalizations (A-3)		
Stereotyped/Idiosyncratic Use of Words or Phrases (A-5)		
Unusual Sensory Interest in Play Material/Person (D-1)		
Hand and Finger and Other Complex Mannerisms (D-2)		
Unusually Repetitive Interests or Stereotyped Behaviors (D-4)		
RRB TOTAL		
OVERALL TOTAL (SA + RRB)		

See the back of this form for guidance on how to convert the Overall Total to the ADOS-2 Classification and the ADOS-2 Comparison Score.

CLASSIFICATION/DIAGNOSIS

ADOS-2 Classification: ______________________

Overall Diagnosis: ______________________

ADOS-2 COMPARISON SCORE
(See back of form for conversion table.)

Level of autism spectrum–related symptoms:	
10, 9, 8	HIGH
7, 6, 5	MODERATE
4, 3	LOW
2, 1	MINIMAL-TO-NO EVIDENCE

W-605B wps. Test with Confidence

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ADOS-2 MODULE 1 23

- STATISTICAL ANALYSES

Differences in mean methylation levels between males and females were evaluated with analysis of variance (ANOVA). Because methylation levels did not show a normal distribution the data were subjected to logarithmic transformation before the analyses. ANOVA was also used to evaluate the correlation among variables. Statistical analyses were performed with the STATGRAPHICS 5.1 plus software package for Windows, and Bonferroni's corrected p-values < 0.05 were considered statistically significant.

RESULTS

GENE-SPECIFIC METHYLATION ANALYSIS (MS-HRM)

Figure 6 shows an example of the melting curves obtained using the standard DNA with known methylation percentages (0%, 12,5%, 25%, 50%, 75%, 100%).

Fig. 6 Standard DNA melting curve

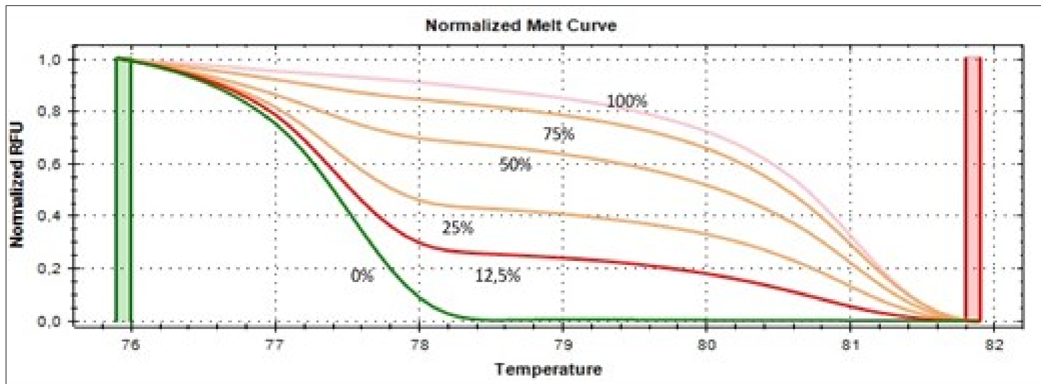


Fig.7 shows an example of the melting curves obtained using standard DNA and a sample of interest.

Fig.7 Standard DNA and a sample melting curves. The arrow indicates the sample showing a degree of methylation between 0 and 12.5%.

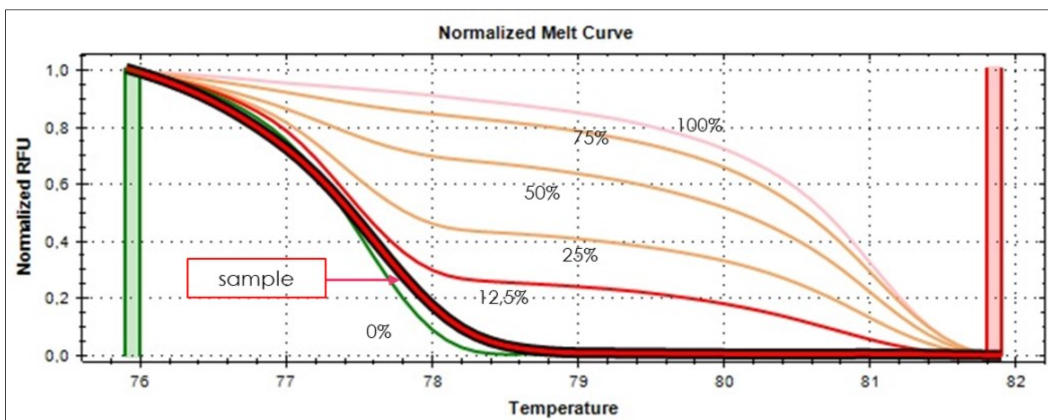


Table 7 summarizes the average levels of methylation of the genes analyzed by MS-HRM.

Table 7. Average methylation levels (%) of the analyzed genes

GENE	MEAN ± SD GIRLS	MEAN ± SD BOYS	*p*-VALUE
OXTR	0.43±0.61	0.07±0.15	0.04*
BDNF	0.31±0.66	0.74±1.17	0.42
RELN	0.27±0.35	0.94±1.00	0.002*
5-HTR1A	1.36±1.06	1.14±0.59	2.38
MECP2	31.68±6.16	7.96±6.11	0.0000*
BCL-2	1.33±1.23	1.10±1.18	3.22
EN2	0.54±1.05	1.39±1.57	0.07

*statistically significant difference between males and females. Bonferroni's correction has been applied to the *p*- values.

Comparing the methylation levels of the analyzed genes between males and females revealed statistically significant differences (Table 7). As expected, being an X-linked gene, methylation levels of *MECP2* were found to be significantly higher in girls than in boys (31,68±6.16% vs 7,96±6.11%; p = 0.0000). However, the *OXTR* gene also showed significantly higher methylation levels in girls than in boys (0.43±0.61% vs 0.07±0.15%; p = 0.04). In contrast, for the *RELN* gene, methylation levels were higher in boys than in girls (0.27±0.35% vs. 0.94±1.00%; p = 0.002).

Tables 8 and 9 show the percentage data extrapolated from the questionnaire administered to the mothers.

Table 8. Data relating to the intake of drugs or supplements extrapolated from the survey administered

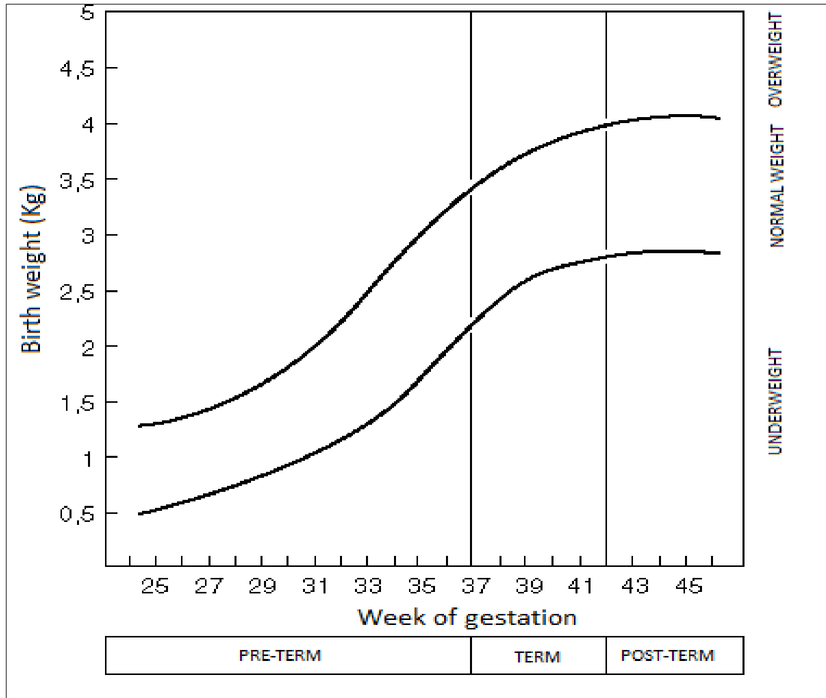
	MOTHERS OF GIRLS		MOTHERS OF BOYS	
	YES	NO	YES	NO
ASSUMPTION OF PARACETAMOL DURING PREGNANCY	(N =15) 36%	(N = 27) 64%	(N = 7) 29%	(N = 17) 71%
ASSUMPTION OF VALPROIC ACID	-	(N = 42) 100%	-	(N = 24) 100%
SUPPLEMENTATION OF FOLATE	(N = 35) 83%	(N = 7) 17%	(N = 20) 84%	(N = 4) 16%
OTHER SUPPLEMENTS	(N = 23) 55%	(N = 19) 45%	(N = 15) 62.5%	(N = 9) 37.5%

Table 9. Data related to maternal factors extrapolated from the administered survey

	MOTHERS OF GIRLS			MOTHERS OF BOYS		
GESTATIONAL AGE	PRE-TERM <37 WEEKS	AT TERM 37-42 WEEKS	POST-TERM >42 WEEKS	PRE-TERM <37 WEEKS	AT TERM 37-42 WEEKS	POST-TERM >42 WEEKS
	(N = 4) 9%	(N = 38) 91%	(N = 0) 0%	(N = 4) 17%	(N = 20) 89%	(N = 0) 0%
BIRTH WEIGHT*	UNDERWEIGHT <2500G	NORMAL WEIGHT 2500-4000G	OVERWEIGHT >4000G	UNDERWEIGHT <2500G	NORMAL WEIGHT 2500-4000G	OVERWEIGHT >4000G
	(N = 1) 2%	(N = 37) 88%	(N = 4) 10%	(N = 2) 8%	(N = 17) 71%	(N = 5) 21%
TYPE OF DELIVERY	NATURAL	CAESAREAN		NATURAL	CAESAREAN	
	(N = 21) 50%	(N = 21) 50%		(N = 11) 46%	(N = 13) 54%	
GESTATIONAL WEIGHT GAIN (GWG)**	LOW	NORMAL	OVER	LOW	NORMAL	OVER
	(N = 17) 45%	(N = 9) 24%	(N = 12) 31%	(N = 6) 25%	(N = 11) 46%	(N = 7) 29%
MATERNAL PRE-PREGNANCY BMI(KG/M^2)	UNDER WEIGHT <18,5	NORMAL WEIGHT 18,5- 24,9	OVER WEIGHT 25-30	UNDER WEIGHT <18,5	NORMAL WEIGHT 18,5- 24,9	OVER WEIGHT 25-30
	(N = 7) 18%	(N = 22) 57%	(N = 10) 25%	(N = 6) 25%	(N = 15) 62.5%	(N = 3) 12.5%

* *classified considering the gestational age (Fig.8)
**classified according to the values recommended by the IOM taking into account the Pre- pregnancy BMI (Fig.9)

Fig.8 Birth weight classification guidelines



Modified by Sweet AY: "Classification of the low-birth-weight-Infant", in Care of the High-Risk Neonate, ed. 3, edited by MH Klaus e AA Fanaroff Philadelphia, WB Saunders Company,1986.

Fig.9 Recommended weight gain during pregnancy based on BMI

Prepregnancy BMI	BMI (kg/m^2)	Total weight gain (kg)
Underweight	<18.5	12–18
Normal weight	18.5–24.9	11.5–16
Overweight	25.0–29.9	7–11.5
Obese	≥30.0	5–9

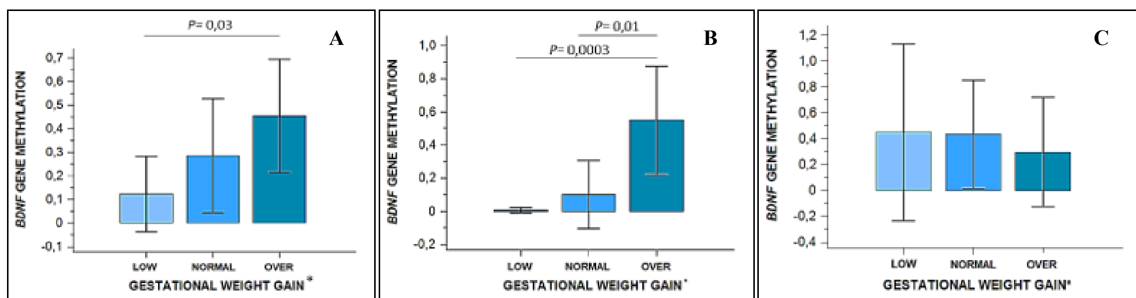
Taken and revised from Rasmussen et al., 2009

CORRELATIONS AMONG VARIABLES

MATERNAL RISK FACTORS AND GENE PROMOTER METHYLATION

The only significant correlation that we observed between the investigated maternal risk factors and gene promoter methylation levels was between the *BDNF* gene methylation levels and maternal gestational weight gain (GWG). The weight gain was classified according to the values recommended by the Institute of Medicine (IOM) taking into account pre-pregnancy BMI. In the total population, *BDNF* gene methylation levels were higher in children born to women who were overweight compared to recommended values (*p*-value= 0.03) (Fig 10 A). After stratification of the ASD population in girls and boys, an even stronger correlation between the *BDNF* gene methylation level and maternal gestational weight gain was seen in girls (*p*-value= 0.0003 between low and over; 0.01 between normal and over) (Fig 10 B), but not in boys (Fig 10 C).

Fig. 10 Correlation between *BDNF* gene methylation levels and GWG. The data are reported as means ± SD. Bonferroni's correction has been applied to the *p*-values. A) Total population; B) Girls; C) Boys.

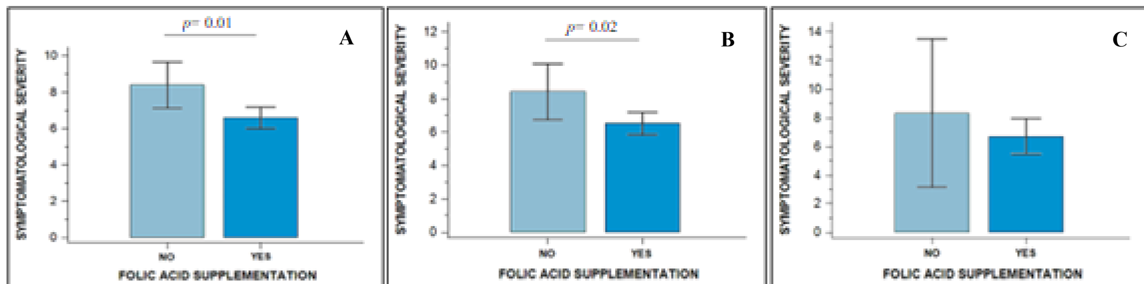


*classified considering the pre-pregnancy BMI

MATERNAL RISK FACTORS AND DISEASE SEVERITY

The only significant correlation that we observed between the investigated maternal risk factors and disease severity was with lack of folic acid supplementation. Figure 11 shows the correlation between ASD severity and maternal folic acid supplementation (before and during pregnancy). It can be seen that, in the total population, the babies of mothers who have not taken folic acid have more severe symptoms than those born from supplemented mothers (*p*-value = 0.01) (Fig 11 A). Similar results were observed in girls with ASD (*p*-value= 0.02) (Fig 11 B), whilst boys showed only a non-significant trend (Fig 11 C).

Fig.11 Correlation between symptomatological severity and Folic Acid supplementation.
Data are reported as means ± SD. Bonferroni's correction has been applied to the *p*-values.
A) Total population; B) Girls; C) Boys.



GENE METHYLATION AND SYMPTOMATOLOGICAL SEVERITY

We evaluated possible correlations between methylation levels of candidate genes and symptomatological severity. Symptomatological severity was classified using the comparison score obtained through a conversion table (Fig. 5). The comparison score ranges from 1, which indicates the minimum degree of severity (Low), to 10 that indicates the highest degree (High). As shown in Table 10, considering the general population, no statistically significant correlations were found. The same results were obtained by stratifying the ASD population into males and females.

Table 10. Correlation between symptomatological severity and gene methylation levels in total population.

	SYMPTOMATOLOGICAL SEVERITY			
GENE	**LOW** (N=9) MEAN ± SD	**MODERATE** (N=30) MEAN ± SD	**HIGH** (N=20) MEAN ± SD	***P*-VALUE***
OXTR	0.11 ± 0.27	0.27 ± 0.36	0.19 ± 0.34	1.35
BDNF	0.35 ± 0.53	0.19 ± 0.41	0.36 ± 0.53	1.29
RELN	0.54 ± 0.48	0.34 ± 0.32	0.25 ± 0.40	0.54
5-HTR1A	0.54 ± 0.38	0.73 ± 0.47	0.78 ±0.45	1.23
MECP2	2.63 ± 0.83	3.11 ± 0.80	2.66 ± 1.03	0.45
BCL-2	0.47 ± 0.50	0.85 ± 0.48	0.62 ± 0.54	0.27
EN2	0.42 ± 0.65	0.45 ± 0.59	0.36 ± 0.54	2.61

*Bonferroni's correction has been applied to the *p*-values.
Gene methylation levels showed a non-normal distribution and therefore are expressed as log-transformed data.

DISCUSSION

ASD are one of the largest groups of complex neurodevelopment disorders defined by 3 clinical features: (1) impairment in social interaction; (2) impairment in communication; and (3) restricted and repetitive patterns of behaviors and interests (Ladd Acosta 2014). They affect about 1-2% of the population with a greater frequency in males, in a ratio of 4.5: 1. The diagnosis of ASD is made on the basis of the clinical observation of the subject and the use of standardized assessment scales, such as ADOS and ADI-R. In recent decades, the prevalence of this disorder has increased significantly and this is thought to be due to both a better understanding of the problem and an improvement in the process and diagnostic criteria. Although numerous studies carried out to date show a considerable variety of causes that can lead to the development of ASD, the etiology of this disorder still remains unknown but it has been shown that environmental, genetic and epigenetic factors play an important role.

In the present study we investigated, in a population of 42 girls affected by ASD, the correlation between the levels of methylation of genes associated with this disorder, maternal risk factors and symptomatological severity. To this end, the experimental protocols for the analysis of 7 genes, namely *MECP2*, *OXTR*, *BDNF*, *5-HTR1A*, *RELN*, *BCL-2*, and *EN2,* were developed using the Methylation Sensitive-High Resolution Melting technique. The anamnestic data were collected through the administration of a questionnaire to the mothers on their lifestyle before and during pregnancy, while the symptomatological severity of the ASD girls were evaluated using the "gold standard" ADOS-2 psychodiagnostic tool. We also recruited 25 ASD boys as comparison

population, in order to assess the presence of any differences between the two genders.

The results obtained from the methylation analyses showed that, except for the *MECP2* promoter, all the other investigated genes showed very low methylation levels, of about 1-2% in average. However, three of the analyzed genes, namely *MECP2* (*p*= 0,0000), *OXTR* (*p*= 0,04) and *RELN* (*p*= 0,002), showed significant differences in mean methylation levels between males and females.

As far as Oxytocin (OXT), this is an important peptide hormone for social cognition and behaviour that mediates its effects through interaction with its receptor (OXTR). Several studies in the literature have examined the role that *OXTR* epigenetic changes may have in the pathophysiology of ASD. The first evidence of an association between *OXTR* methylation and ASD comes from the study by Gregory et al. (2009) where it was described the case of two siblings both affected by ASD, one with a deletion (inherited from the mother) in region 3p25.3 containing the *OXTR* gene, while the other, who had not inherited the deletion, showed a higher methylation at sites CpG - 924 and - 934 (compared to TSS) in the MT2 region of the *OXTR* gene promoter than the father. The same study also analyzed the methylation levels at positions - 860, - 934 and - 959 of the *OXTR* gene promoter in DNA samples extracted from peripheral blood mononuclear cells (PBMC) of 20 individuals with ASD (10 males and 10 females) and 20 healthy controls (10 males and 10 females) and found a significant increase in methylation in individuals with ASD compared to controls. In addition, a similar methylation model was reported by analyzing methylation levels at CpG - 860, - 901, - 924, and - 934 sites of the *OXTR* gene promoter in DNA extracted from *post-mortem* brain tissue samples (temporal cortex) of 8 ASD individuals and 8 healthy controls. In

males (6 of 8 ASD individuals) the increase in methylation levels corresponded to a 20% decrease in *OXTR* mRNA expression level (Gregory et al., 2009). Since methylation at CpG - 934 was significantly different between ASD cases and controls in both peripheral blood and brain tissue samples, the authors hypothesized that the results obtained from methylation analysis performed on peripheral blood samples could potentially serve as a marker for methylation status in brain tissue (temporal cortex) [174]. Likewise, Elagoz Yuksel and collaborators analyzed the levels of methylation of four consecutive regions (MT1, MT2, MT3 and MT4) in *OXTR* gene promoter in peripheral blood samples from 27 children with ASD and 39 healthy controls. Results shown a reduction in methylation only in MT1 and MT3 regions in subjects with ASD, while they found no significant differences within the MT2 area, such as those previously highlighted in the study by Gregory and collaborators [175]. Andari et al. studied the relationship between *OXTR* methylation (Introne 1 and exon 1 of the MT2 region), the diagnosis of ASD, the severity of social symptoms and the relationship between *OXTR* methylation and functional brain connectivity in adults with ASD. First they analyzed, in DNA extracted from saliva samples from 35 subjects with ASD and 64 healthy controls (17 F and 47 M), the methylation levels of the *OXTR* gene reporting higher levels of methylation in intron I of the MT2 region (CpG 16) of the *OXTR* gene promoter in ASD. Hypermethylation was correlated with clinical symptoms and hypoconnectivity of brain areas involved in theory of mind and default mode (superior temporal sulcus and posterior cingulate cortex). Finally, they analyzed methylation levels in exon 1 of the MT2 region and these were positively correlated with social response deficits in ASD and hyperconnectivity between striatal and cortical brain areas. These results suggest that epigenetic changes in the OXT signaling pathway may be associated with

symptom severity and brain connectivity [176]. In this thesis, we analyzed the MT1 region of the *OXTR* gene promoter. The results showed methylation levels close to 0% in all samples with a significant difference between males and females (p = 0.04). To the best of our knowledge, there are no studies in the literature reporting gender differences in methylation levels in the region we considered.

Another protein that plays an important role during neurodevelopment is Reelin, involved in cell positioning and neuronal migration during brain development and in synaptic strength and connectivity in the adult brain [208]. In some diseases such as ASD, Schizophrenia (SZ), Alzheimer's disease (AD), altered RELN levels have been identified that have been associated with a lower density of the dendritic spine. Several studies conducted on *post-mortem* tissue of ASD subjects have reported significant reductions in the RELN protein in the cerebellum [209] and in the upper frontal cortex, parietal cortex and cerebellum of ASD subjects [178]. Similar studies have shown a significant reduction in *RELN* mRNA levels in the frontal cortex and cerebellum of ASD subjects [178]. Similar reductions have been found measuring Reelin plasma levels *in vivo* [210]. A possible mechanism that could explain the reduced expression of *RELN* mRNA in ASD could be the hypermethylation of the *RELN* promoter as occurs in SZ patients. An example is the study of Abdolmaleky e coll. that evaluated *RELN* promoter methylation levels in 5 *post-mortem* brain tissue samples from patients (male) with SZ and 5 healthy controls (male). The results showed a significantly higher level of methylation in subjects with SZ than in controls. They also analyzed *RELN* expression levels and found an inverse relationship between the level of DNA methylation and the expression of the *RELN* gene. This evidence supports the theory that DNA methylation of the *RELN* promoter influences gene expression and therefore may participate in the

aetiology and pathogenesis of Schizophrenia [211]. Another study, which examined DNA methylation levels of the *RELN* promoter was carried out on peripheral blood samples of 110 SZ subjects and 122 healthy controls. Also in this case, the results showed a significantly higher level of methylation of the *RELN* promoter in patients with SZ compared to controls. Then, the effect of DNA methylation on *RELN* gene expression was evaluated, finding a decrease in expression in samples with a higher level of methylation compared to those with a lower level of methylation. Finally, it is important to note that in this study differences in methylation levels between males and females were found, with higher methylation levels in males than in females [212]. To date few studies have examined the levels of methylation of the *RELN* gene in subjects with ASD, an example is the study carried out by Lintas and coll., on *post-mortem* brain tissue samples of 6 post-puberal ASD subjects (5 males and 1 female) and their healthy controls. This study shows a difference in methylation pattern between ASD patients and healthy controls. In particular, the upstream region of the promoter results methylated specifically in ASD brains while a downstream region is methylated only in controls. The results of this study also confirmed the presence of reduced levels of *RELN* mRNA in ASD brains compared to controls. The authors conclude by assuming that ASD-specific CpGs, located in the upstream region of the gene promoter region, may play a functional role in the risk of ASD by reducing the expression of the *RELN* gene [179]. To our knowledge, ours is the first study that analyzed *RELN* gene methylation levels in peripheral blood samples from ASD subjects. Our results showed demethylation of the promoter region of the gene in all analyzed samples with a significant difference (p = 0.002) in methylation levels between males and females.

Aberrations of the serotonergic system seem to be involved in the pathophysiology of neurodevelopmental disorders including ASD. High levels of serotonin (5-HT) in platelets were the first observation that related 5-HT to ASD. In particular, hyperserotoninemia was observed in about 25% of patients with ASD. In addition, it was seen that a variation in the expression of the *5-HTR1A* gene (5-HT receptor) may have a potential role in the development of ASD. The study carried out by Yahya and collaborators in 2019, on RNA samples isolated from whole blood of 30 individuals with ASD (18 males and 12 females) and 20 healthy controls, reported a reduction in *5-HTR1A* gene expression levels in the affected patient group compared to controls [191]. There are no known studies in the literature that have evaluated the methylation levels of the *5-HTR1A* gene in subjects with ASD. In this thesis work, methylation levels of this gene were analyzed in samples of DNA extracted from peripheral blood of children with ASD and reported demethylation in all samples tested without differences between males and females.

BCL-2 (B-cell lymphoma 2) is an apoptosis regulatory protein [193] and its gene has been linked to ASD [194]. Studies have reported a decrease in *BCL-2* expression in the cerebellum and frontal cortex of subjects with ASD compared to healthy controls matched for age and sex [195,196]. Another study analyzed DNA from lymphoblastoid cell lines derived from B-cell (LCL) of three pairs of monozygotic twins that differed in the severity of ASD and their non-autistic siblings. Methylation profiling revealed many candidate genes differentially methylated between discordant MZ twins as well as between both twins and non-autistic siblings. Cross-referencing these results with the expression analysis of differentially expressed genes in the same samples it was found that most of the genes showed inverse correlations between methylation levels and

expression, confirming the role of epigenetic regulation of gene expression through differential DNA methylation [197]. In this thesis work the analysis of the methylation levels of the promoter of this gene showed demethylation in all samples tested without significant differences between males and females.

Another gene analyzed in this thesis was *EN2* (Engrailed-2), a transcription factor that is considered an ASD susceptibility gene based on neuroanatomical similarities between ASD and abnormalities of cerebellar development in rodent models. In mice, *En2* is highly expressed in Purkinje cells during fetal and early postnatal development, where it acts primarily as a transcriptional repressor until it is downregulated during the perinatal period. It is also expressed in neurotransmitter systems that have been implicated in autism such as serotonin. En-2 - /- knockout mice show a reduction in cerebellum size and cell count, with a reduction of about 30-40% of major brain cell types including Purkinje cells [198]. Although human studies are limited, expression analysis in fetuses at 40 weeks of gestation has indicated widespread gene expression of *EN2* in brain regions including cerebellar cortex and deep nuclei. Some studies report reductions in the number of Purkinje cells in the brain of subjects with ASD that appear to occur in the late prenatal period coinciding with downregulation of *EN2* [199]. James et al. conducted a study on *post-mortem* cerebellar samples of 13 individuals (4 F + 9 M) with ASD and 13 healthy controls and found higher levels of *EN2* promoter methylation in affected individuals than in controls. They also found a significant increase in *EN2* gene expression and protein levels. These results may suggest that normal *EN2* downregulation, which signals the maturation of Purkinje cells during late prenatal and early-postnatal development, may not occur in some individuals with autism and that the presence of postnatal *EN2* overexpression may contribute to the

cerebellar abnormalities found in ASD [200]. The analyses of the promoter of this gene carried out in this thesis work, using the MS-HRM technique, showed demethylation in all the samples analyzed.

Several lines of evidence suggest that the MECP2 protein plays an important role in ASD. The *MECP2* gene encodes MECP2 (methylated CpG-binding protein 2), a transcriptional repressor expressed in many tissues that binds to methylated CpG and that plays an essential role in neuronal development. Alterations in physiological MeCP2 levels caused by over-expression due to gene duplication (MeCP2 duplication syndrome) or loss of expression due to mutations (Rett syndrome) have been associated with social behaviour disorders similar to those found in ASD [213,214]. Recently, mutations in *MECP2* have also been identified in ASD patients [215]. In addition, *MECP2* expression has been found significantly reduced in 11 *post-mortem* frontal cortex samples of individuals with ASD compared to healthy controls. This reduced expression was then correlated with an increase in the DNA methylation of the promoter of that gene [172]. A further demonstration of the important role of *MECP2* promoter methylation in the aetiology of ASD, came from a very recent study, carried out on mice, which showed how the locus-specific DNA methylation of the *Mecp2* promoter, obtained using a methylation targeting method based on dCas9, correlated with a reduction in the expression of this gene and a number of behavioural alterations, including reduced social interaction, increased anxiety/depression and poor performance in memory tasks, similar to those found in ASD [216]. To the best of our knowledge this thesis work is the first to analyze the promoter methylation levels of this gene in DNA samples extracted from peripheral blood of children with ASD and methylation levels were found to be (Mean ± SD) 31,68 ± 6,16% in girls and 7,96 ±

6,11% in boys. The explanation for this clear difference (*p*= 0.0000) could be found in the location of the *MECP2* gene. This gene is in fact located on the X chromosome and is therefore subject to the lyonization process, a mechanism that allows the phenomenon of compensation of the gene dosage between females (XX) and males (XY). Methylation plays a key role in the inactivation of the X chromosome and this could explain the high levels of methylation found in the population of females compared to that of males.

Another gene taken into consideration in this work was the *BDNF*. The analysis of its methylation levels reported a demethylation in all samples tested without significant differences between males and females. BDNF is a member of the neurotrophic family that is involved in the survival and differentiation of dopaminergic neurons in the developing brain and plays an important role in the formation and plasticity of synaptic connections. It has been hypothesized that synaptic dysfunction may be a possible mechanism for the onset and progression of neurodevelopmental disorders. A support to this hypothesis comes from the evidence that characteristics of ASD are often found in patients with genetic diseases (for example, the X-fragile syndrome), where alterations in synaptic function have been found [217]. In addition, patients with ASD often show a dysregulation of proteins associated with synaptic plasticity, such as BDNF (brain-derived neurotrophic factor). A study, based on a murine model, investigated the induction of *Bdnf* expression by valproic acid (VPA), a drug whose intake during pregnancy is strongly related to ASD both in the murine model and in humans. The results showed that the administration of VPA to pregnant mice (gestational age 12.5 days), which is known to cause autistic symptoms in offspring, led to a transient increase in both mRNA and BDNF protein levels of about 5-6 times in the

fetal mouse brain. Given the well-established role of BDNF in regulating neurogenesis during early embryonic development [218,219], alterations in its expression, in the fetal brain, can lead to VPA-induced cognitive disorders altering brain development [220]. Among the various mechanisms through which VPA induces alterations in BDNF expression, in the mouse model, the most likely one seems to be modification in epigenetics mechanisms. In this regard a recent study has investigated, in samples of fetal brain tissue from mice (male and female), the induction of *Bdnf* expression by VPA as well as the underlying epigenetic mechanisms. They initially analyzed the effect of VPA on the expression of Bdnf exon 9 (Ex9) and found an increase in mRNA of this gene in both males and females. They also analyzed the expression of Ex1, Ex4 and Ex6 exons. In this case, for Ex1 and Ex4 exons the effect of VPA on expression was sexually dimorphic, in fact there was a greater expression in females than in males. The authors hypothesized that the stimulatory effects of VPA on *Bdnf* transcription could be mediated by epigenetic mechanisms such as changes in acetylating levels of histones within and upstream of Ex1, Ex4 and Ex6 exons and changes in methylation levels of CpG sites in the promoters upstream of the exons considered. They then analyzed the acetylation levels of H3K9/14, H3K27 and H4K5/8/12/16 histones and found an increase in acetylation consistent with all sites examined. Regarding the methylation levels of the CpG sites considered, no differences were found in DNA methylation levels, a sign that these changes do not mediate the effects of VPA on *Bdnf* in the developing brain [221]. As far as human studies, a study carried out by Taurines and collaborators, reported a reduction in mRNA levels of *BDNF*, extracted from whole blood, in patients with ASD compared to healthy controls combined by age and gender [187]. A possible mechanism that could explain this reduction in expression is an

alteration in methylation levels of this gene. In this regard, a study carried out by Wang and colleagues on DNA extracted from peripheral blood cells from 5 children diagnosed with ASD (DSM-IV) and 5 healthy controls reported the presence of over 200 genes significantly methylated between cases and controls. In particular, compared to the control group, 19 genes were found to be hypermethylated in the affected group, including the promoter of the *BDNF* gene[188].

In this thesis work methylation levels of the genes analyzed were correlated with the maternal factors taken into consideration. Considering only the case studies of females (N=42), the results showed a statistically significant association between *BDNF* gene methylation levels and weight gain during pregnancy. In particular, we found higher levels of methylation in girls whose mothers showed a higher weight gain than recommended by the Institute of Medicine (IOM) taking into account pre-pregnancy BMI. However, these are slight increases so subsequent studies will be necessary to assess whether or not these values correspond to changes in gene expression. This correlation has not been observed in the case studies of males, perhaps due to the small sample size, but remains in the total population (M+F) and deserves further investigation.

Intrauterine exposure to high maternal adiposity or high gestational weight gain (GWG) has been associated with adverse fetal development and, under the assumption of the origins of health development and disease (DOHaD), may affect offspring's health even in old age. Recent studies have found an increased risk of ASD in children of mothers with excessive GWG. An example is the study performed by Windham and colleagues carried out on a cohort of children born between 2003 and 2006 in different U.S. countries and their mothers. The children were evaluated by

means of special tests and classified according to the presence or absence of ASD, while the mothers were given a telephone interview in which they were asked for information regarding their lifestyle in the pre-pregnancy period and during pregnancy. The association analysis carried out in this study reported a significant correlation between high weight gain in pregnancy and the risk of ASD in offspring [140]. Other studies in the literature report results in line with those mentioned above [139,141,222]. To date it is not yet clear whether the observed effects are attributable to direct intrauterine effects, caused by maternal overweight or whether they can be explained by environmental factors. In recent years several hypotheses have been made on what may be the mechanisms that link maternal obesity with the neurological development of offspring. One of the hypotheses concerns the alterations of epigenetic mechanisms. In support of this hypothesis, a 2013 study carried out on DNA samples extracted from peripheral blood of siblings aged between 2 and 25 years born from obese mothers before and after bariatric surgery, and therefore with associated weight loss, has reported several CpG sites differentially methylated [223]. In addition, three cohort studies have found associations between maternal BMI and offspring DNA methylation at birth [224,225] and at 3 years of age [226]. In the study carried out by Sharp et al. were found several sites of CpG differently methylated in the umbilical cord blood of children of obese and underweight mothers compared to children of mothers of normal weight. In particular, a comparison between the offspring of women with normal weight and the offspring of obese women identified 28 differentially methylated genes including *BDNF* which showed higher levels of methylation in the offspring of obese mothers [225]. BDNF is a target of the miR-210, which reduces its level of expression. This miRNA was found overexpressed in

placental samples of women with high pre-pregnancy BMI [227]. These observations have suggested an association between pre-pregnancy BMI and epigenetic changes (methylation and miRNA) that lead to alterations in *BDNF* gene expression levels.

Another statistically significant correlation found in this thesis, both in the female and in the total population, was that between the intake of folic acid during pregnancy and the ASD severity. In particular, a greater symptomatological severity was found in children whose mothers had not taken folic acid during pregnancy. In recent decades, it has been seen how the administration of drugs to mothers during pregnancy can have harmful effects on fetal development. Among the drugs with the highest teratogenic potential we find those used for the treatment of neurological diseases such as antiepileptic drugs (AED). It is known that some substances can help prevent the development of neurodevelopmental diseases including ASD. One of these is folic acid, which has a very important role in ensuring proper neurological development. Around the '30s Wills et al. conducted research on a particular form of anemia (tropical megaloblastic anemia) that affects women during pregnancy, discovering that this was caused by a deficiency of folic acid [228]. Since then this form of anemia was treated with folic acid supplementation during pregnancy and this led, around the 60s, researchers to discover how maternal folic acid supplements had a protective effect against NTDs (Neural tube defects) [229]. After this discovery folate supplements were recommended for women who were pregnant or planning to become pregnant in several countries and regions. In 2006, the WHO approved the recommendation that women of reproductive age should take 400 µg of folic acid per day in order to reduce the risk of NTDs [230]. Folate deficiency during the periconceptional period has been also associated with an increased risk of neurological developmental disorders such as ADHD [231] and

ASD [232]. A 2012 study of a cohort of mother-child couples reported an association between folate intake during the first month of pregnancy and a lower risk for offspring to develop ASD [154]. Another 2013 study of children born in Norway between 2002 and 2008 reported that in children whose mothers had taken folic acid during pregnancy only 0.10% had developed ASD compared to 0.21% in those whose mothers had not. So again, folic acid supplementation during pregnancy was associated with a lower risk of ASD [156]. Finally, the study carried out by Tan and collaborators (2020) showed that out of a total of 617 children with ASD, those born to mothers who had not taken folic acid or other nutritional supplements during pregnancy, had more severe cognitive and social alterations as well as communication deficits, autistic behavioural mannerisms, delays in the development of motor behaviour more pronounced than children born to mothers who had taken folic acid and supplements during pregnancy. This study is an excellent example of how non-supplementation with folic acid in the pre-pregnancy period can be an explanation for the worsening of the diagnosis [233]. However, other studies have not found a statistically significant association between folic acid and ASD [234]. The discrepancy between these results suggests that it is probably important not only to take folates, but to take them at a specific time of pregnancy and in the right doses [235].

In this thesis work we found statistically significant differences in the methylation levels of three of the genes analysed between girls and boys. While the differences in methylation of *MECP2* gene (X-linked gene) can be explained by inactivation of one of the two X chromosomes in females, with regard to specific sexual methylation of autosomal loci the causes are not yet clarified. In the literature there are several studies on DNA methylation of healthy subjects in which gender differences are found.

An example is the study by Sarter et al. (2005) in which were studied the levels of methylation of the promoter regions of four autosomal loci (*MTHFR*, *CALCA*, *MGMT* and *ESR1*) in DNA extracted from whole blood of 134 healthy males and 157 females observing statistically significant differences between male and female methylation levels. In particular, three of the four loci analyzed showed a higher methylation for males, while *ESR1* methylation levels were not gender dependent [236]. Another study is the one conducted by Liu et al., in which DNA methylation levels were examined in saliva samples from 197 healthy subjects (54 females) at about 20,000 CpG sites. The results showed that gender influences not only the methylation of X-linked genes, but also of autosomes, in fact about 500 autosomal sites showed strong differences between males and females [237]. The study conducted by Singmann et al., in 2015 compared the levels of autosomal DNA methylation between males and females in blood samples from 1799 healthy subjects and 3 other independent cohorts used to replicate the results. The results showed 1184 CpG sites that were differentially methylated between males and females and these sites were found to be distributed across all autosomes. In addition, some of the methylated loci showed differential gene expression between males and females [238]. Finally, in the study by Maschietto et al., in 2017, DNA methylation levels were analyzed in cord blood samples from healthy newborn babies (39 females and 32 males), born at term and with a normal birth weight. The results reported approximately 2,000 differentially methylated CpG sites (DMS) between the sexes [239]. These results were compared with those obtained from another study in which DMSs were analyzed in brain tissue samples from patients with schizophrenia and healthy controls. The analysis reported an overlap of 116 CpG sites

between the two studies, indicating an enrichment for schizophrenia-related CpG sites that differ between females and males in cord blood [240].

Some studies have reported gender differences in methylation levels in subjects suffering from diseases that show a gender discrepancy in terms of severity and/or susceptibility such as cardiovascular disease (CVD) and neurodevelopmental disorders. A differential methylation of genes associated with CVD and involved in aging, lipid metabolism and blood lipid levels has been reported. For example, methylation of *CETP*, *LPL* and *PLTP* gene promoters was found to be higher in females than in males. In contrast, the *ABCG1* gene was more methylated in men [241]. With regard to ASD, a recent study examined the methylation levels of the *HTR4* gene promoter in DNA samples extracted from peripheral blood of 61 (50 M and 11 F) subjects with ASD (age about 4 years) and 66 healthy subjects (age about 6 years), reporting significantly lower methylation levels in subjects with ASD compared to controls. In addition, methylation levels were significantly higher in females than in males among subjects with ASD, while among control subjects this gender difference was not found [242].

To our knowledge, our study is the first to find gender differences in the methylation levels of the *MECP2*, *OXTR* and *RELN* genes in blood DNA from subjects with ASD.

One of the biggest obstacles in finding epigenetic markers in brain-related disorders such as ASD is the difficulty in sourcing brain tissue, which is why many studies use peripheral tissues such as blood. Epigenetic patterns necessarily differ between tissue types, resulting in the fact that methylation observed at the peripheral level may not necessarily reflect that present at the brain level. However, an important advantage of using peripheral tissues, in addition to easier availability, is to be able to study the molecular events associated with these disorders in the living subject, whereas the

study of *post-mortem* brains may not reflect the epigenetic pattern of brain tissue in a still-living person. In this sense, a 2016 work [243] analyzed through an epigenome-wide approach the DNA methylation levels extracted from temporal lobe biopsies and peripheral blood of living subjects with epilepsy. The results showed that only about 8% of the CpG sites analyzed showed high levels of correlation between the methylation levels of the two tissues. These results suggested that although most DNA methylation markers in peripheral blood do not completely reflect the DNA methylation status of the brain, there may still be peripheral markers whose methylation status may coincide with that of the brain tissue and may allow us to identify significant epigenetic differences in different brain disorders.

Strengths of the present study include the relatively large number of phenotypically well-characterized female ASD subjects and the availability of a comparison population consisting of male ASD subjects that allowed us to assess any gender differences. The main limitation of the study is that, due to the unique situation that we have experienced during the COVID-19 pandemic, it was not possible to recruit a control population so that present results should be considered preliminary and a further studies are required, including the investigation of healthy children, to clarify if the observed sex-related methylation differences can reflect ASD-specific biomarkers and be involved in the gender discordance observed in ASD. A better understanding of the molecular bases involved in these gender differences could help to develop more targeted therapies with higher success rates, especially in diseases such as ASD where these differences are more pronounced.

In conclusion, this thesis work can be considered a starting point for further studies, to be carried out on a larger cohort including a control population, in order to confirm the results obtained and give more support to the hypothesis that epigenetic and environmental factors play an important role in the aetiology of autism spectrum disorders.

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