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Genetic and phenotypic characterization of Autism Spectrum Disorders

PhD Tutor: Prof. Renato Borgatti

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Silvia Annunziata

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Sommario

1.Introduction

1.1. Foreword

Autism spectrum disorder (ASD) is a Neurodevelopmental Disorder characterized by deficits in social behaviors and communication, restricted interests, and repetitive patterns of behavior (American Psychiatric Association, 2013).

The overall prevalence of ASD is estimated to be 18.5 per 1,000 (one in 54) in children aged 8 years, varying from 13,1 to 31,4 per 1000 among the CDC-established Autism and Developmental Disabilities Monitoring (ADDM) network sites (Maenner MJ et al., 2020).

ASD typically manifests in the first years of life with a wide range of clinical presentation and is persistent throughout life.

1.1.1 Diagnosis

Diagnosis of ASD remains a clinical diagnosis that requires a multidisciplinary approach. It is based on clinical criteria; the instrument most accredited and used is the Diagnostic and Statistical Manual of Mental Disorders (DSM–5) (Table 1).

The two core elements of the diagnostic process of autism in children are: a detailed developmental history, that is usually obtained from parents and should cover both early history with first concerns and the actual clinical problems, and an observation of the child's interactions with their parents and with unfamiliar adults during a combination of structured and unstructured assessments.

Table 1 – ASD criteria according to DSM-5

The Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) criteria for autism spectrum disorder (ASD) comprise five symptom clusters (A–E).
A. Social communication and social interaction
• **Must have evidence across multiple contexts of all of the following three subdomains currently or by history:**
• Social reciprocity
• Non-verbal communication
• Developing, maintaining, and understanding relationships
B. Restricted, repetitive behaviors and interests
• **Must have evidence of two of four of the following subdomains currently or by history:**
• Stereotyped, repetitive behaviors
• Insistence on sameness
• Highly restricted, fixed interests
• Hypersensitivity or hyposensitivity or interest in sensory inputs
C. Symptoms must be present in early development but may not fully manifest until later or may be masked later in life by learned strategies
D. Symptoms must cause clinically significant impairment in current functioning
E. Not better explained by intellectual disability or global developmental delay
Note: previously established DSM-IV diagnoses of any pervasive developmental disorder, including Asperger's disorder, should be assumed to be equivalent to DSM-5 ASD. ASD may co-occur with many other disorders, including attention-deficit/hyperactivity disorder, intellectual disability, language delay and genetic syndromes.

The diagnostic process should therefore include an accurate anamnestic collection that considers the aspects related to familiarity, pregnancy and the perinatal era and the main stages of psychomotor development, a profile of adaptive skills with standardized tools (i.e. Vineland Scales), and a cognitive or psychomotor developmental assessment. In children, the mode of parent-child interaction and parent coping strategies should also be investigated, as they are useful in planning

an appropriate therapeutic intervention. It is also important to collect information related to the functioning of the subject in other areas outside the family, such as the degree of interaction with peers or behavioral and relational aspects in the main educational contexts. Alongside these aspects, an adequate objective and neurological examination should be conducted, to detect abnormalities in stature or ponderal growth, minor physical abnormalities, dyschromia, or any neurological deficits.

Diagnostic tools addressed specifically to core symptoms are many and must be chosen and used carefully, because they can in no way substitute the clinician for an accurate diagnostic process.

Several structured diagnostic interviews and observational assessments for autism exist, but only a limited number have been rigorously tested for diagnostic accuracy relative to the gold standard of expert clinician judgement.

The most used checklist is the CARS (Childhood Autism Rating Scale) (Schopler E et al, 2010). This measure consists of a 15-item structured interview, with each item scored according to seven levels of severity. The scale was designed to be used for children older than 2 years, requires training to administer and it allows, based on the score obtained, to classify autism as mild or moderate-severe. It is still "the strongest, best-documented, and most widely used clinical rating scale for autism, even if it may overidentify very young children or those with severe mental retardation and may under identify older patients with high-functioning autism.

Autism Diagnostic Interview-Revised (ADI-R) is a clinical tool for the diagnosis of Autism in children and adults. It consists of a standardized and semi-structured clinical interview that, through 93 voices, analyzes the child's behavior by investigating the quality of reciprocal social interaction, communication and language, and the presence of restricted, repetitive, or stereotyped interests or

behaviors. (Lord C, 1994). ADI-R is appropriate for children and adults over 18 months of mental age.

The ADOS (Autism Diagnostic Observation Schedule), of which the second version is available (Lord, 2012), is instead a semi-structured evaluation of play, which investigates the communicative qualities, social interaction, and the presence of symbolic play in children and adults suspected for ASD.

Although these interviews and assessments have reasonably robust sensitivity, specificity and reliability and are widely used in some services in communities, there are also challenges to the widespread adoption of the best validated instruments: the Autism Diagnostic Interview-Revised (ADI-R) (Lord C, 1994) and the Autism Diagnostic Observation Schedule-2nd Edition (ADOS-2) (Lord, 2012). These challenges include the cost of the instruments and training, the time required to complete them and the need for substantial training to use them reliably

As the diagnosis of ASD was previously made mainly after 3-4 years of life, the increased awareness and the use of screening methods in the first years of life is augmenting early diagnostic rate.

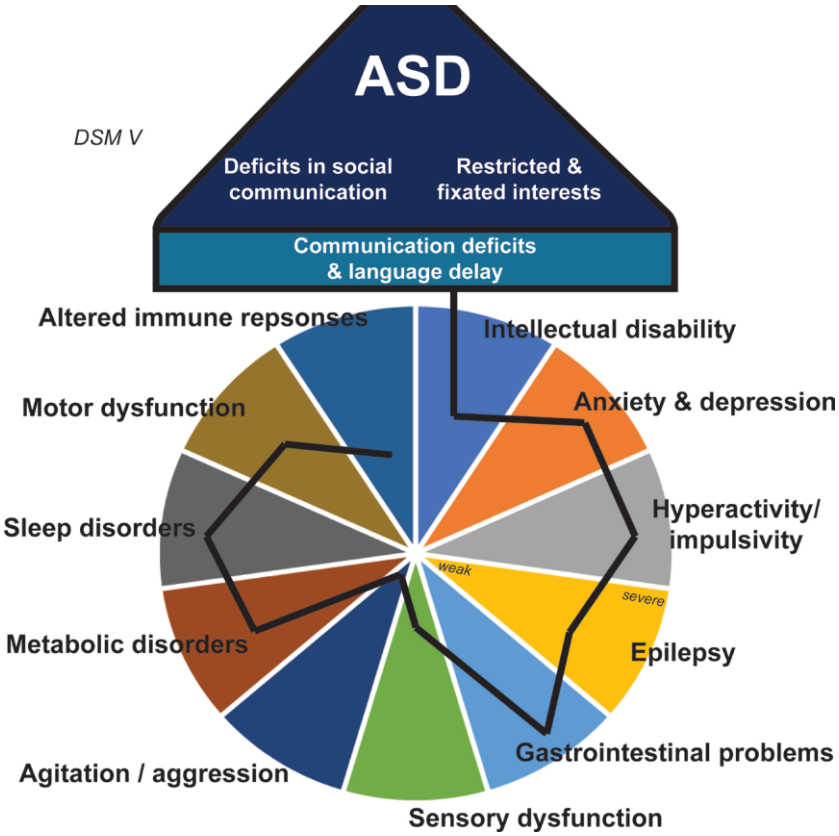
The stability of a diagnosis of autism from the preschool years to mid-childhood is relatively high (Lord et al, 2009). However, although diagnostic systems currently presuppose that autism is a lifelong condition, there is a growing recognition that autism has a heterogeneous developmental time course (Georgiades, S et al, 2017).

Early recognition and early intervention are in fact the unique ways till now available to guarantee the best outcome for these children.

1.1.2 A spectrum of clinical heterogeneity

ASD diagnosis can be sometimes challenging, because it is a real heterogeneous condition, because the clinical presentation can vary not only during time-course but with huge differences among different behavioral phenotypes. DSM-5 tried to

Figure 1 - ASD heterogeneity in clinical presentation (Sauer et al, 2021)



obviate to this heterogeneity with some specifiers (i.e. intellectual and/or language impairment, association with a known medical or genetic condition or environmental factor; association with another neurodevelopmental, mental, or behavioral disorder)

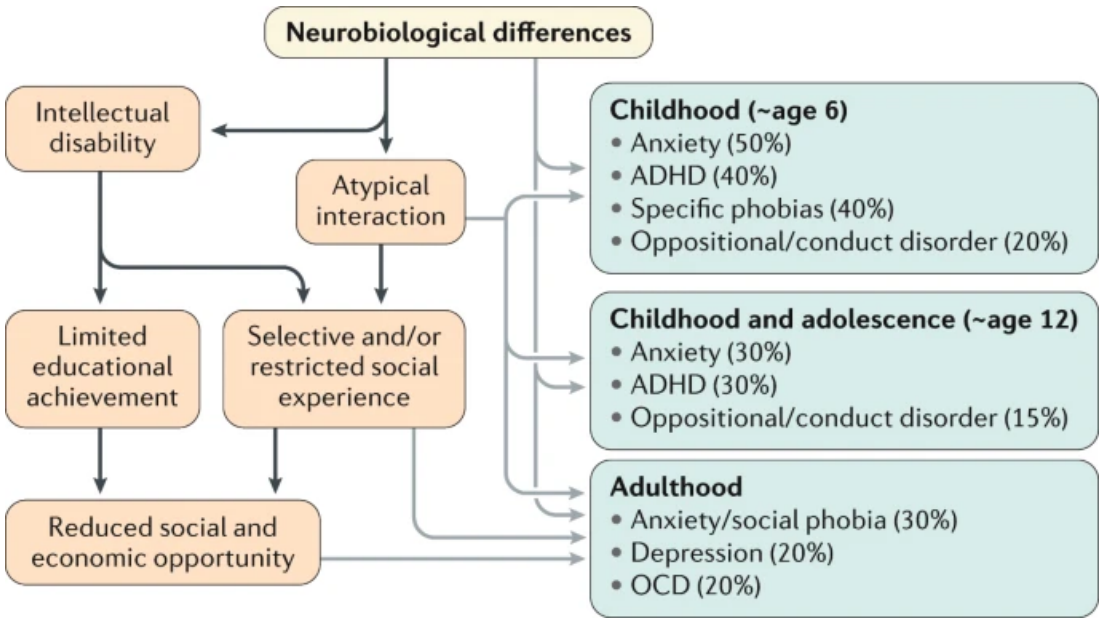
Considering only the core symptoms we can in fact observe a spectrum of clinical presentation.

However, this heterogeneity is due not only to differences in core deficits, but also to comorbidities or associated clinical issues (see Figure 1). In fact, in ASD we can often find associated symptoms, and it is well established that psychiatric, neurodevelopmental, or neurologic comorbidities are the rule than the exception, and these aspects often strongly affect daily functioning and adaptation skills of ASD subjects, sometimes more heavily than the core symptoms themselves. In fact, more than 70% of individuals with ASD have a concomitant medical, psychiatric, or associated neurodevelopmental disorder, and about 40% of subjects have two of them. As showed in figure 1, all these aspects, taken together, strongly affects the different functioning profiles and the ways of presentation of the disorder.

The aspect even more challenging is the change over time of intraindividual presentation, also known as "chronogeneity" (see Figure 2)

For ASD preschool children the most frequent co-occurring conditions and comorbidities are language delays, motor problems, epilepsy, difficulties with sleep and eating, and high levels of activity (Soke et al, 2018). In school age children years ADHD, anxiety, obsessive–compulsive disorder (OCD), intellectual disability, irritability, and disruptive behaviors occur more frequently. The proportion of individuals with depressive symptoms becomes higher in adolescents and adults (Pezzimenti et al, 2019). Adults with autism are more likely to be diagnosed with many physical health conditions than adults in the general population, such as immune conditions, sleep disorders and obesity.

Figure 2 - "Chronogeneity" in ASD comorbidities (Lord et al, 2020)



These difficulties and disorders taken together contribute to ASD severity as well as they affect the level of independence and adaptive functioning (Lord et al, 2020).

This heterogeneity is a diagnostic challenge, both for diagnosing comorbidities, and for differential diagnosis, and it is a risk for clinicians, who could not recognize, and adequately treat, an associated disorder, for the phenomenon known as

"overshadowing". In these terms clinicians risk to ascribe all behavioral manifestations to the principal diagnosis, and not to analyze them according to other possible other explanations.

1.1.3 Complex and essential ASD

As above discussed, ASD is a clinical diagnosis, but growing literature demonstrates that it is neurobiological disorder; whose etiopathogenesis remains in most cases still unknown. The clinical heterogeneity of ASD has made categorization difficult but it suggests the presence of different endophenotypes that indicates possible different underlying etiologic factors. Miles and colleagues proposed the classification in complex and essential ASD (Miles and Hillman 2000; Miles et al. 2005). Complex ASD describes individuals with six or more minor anomalies and/or systemic congenital malformations, such as congenital heart defects (Ingram et al. 2008), while essential ASD describes children with ASD without facial peculiar characteristics and/or different kind of malformations (Miles et al. 2005). Complex ASD includes the 20-30% of ASD subjects, has a worse prognosis and a male to female ratio of 2,5:1. (Ingram et al., 2008), while essential autism represents the remaining 70-80% of ASD subjects; this group has a higher male to female ratio (4,5:1) and more frequent familiarity for ASD and neuropsychiatric disorders (Devlin and Scherer, 2012).

Complex ASD is often associated with chromosomal abnormalities or monogenic alterations.

1.1.4 Genetics

All these considerations have led over time a growing interest in ASD genetic studies.

First of all, the heritability of ASD is supported by studies demonstrating risk for ASD recurrence in 11-19% of families with one affected child compared to 1-2% risk for ASD in US population (Ozonoff et al., 2011; Ronald & Hoekstra, 2014). In families with existing cases of ASD, familial clustering can be observed. Younger siblings of family members with an ASD diagnosis face an increased risk for ASD, even more so for younger male siblings (Sauer et al 2021).

Moreover, twin studies revealed high concordance rates for ASD in monozygotic twins (88%–95%) compared to dizygotic twins (31%) (Rosenberg et al., 2009), giving further support to the important role of genetics.

Approximately 10% of individuals with ASD has a defined Mendelian condition or genetic syndrome, such as Fragile X syndrome (1-2% of ASD cases), tuberous sclerosis (1%), Rett syndrome (0.5%) and Neurofibromatosis (NF1 <1%). Other rare microdeletions or single gene defects associated with ASD include Williams–Beuren, Sotos, Cowden, Moebius, Smith-Lemli-Opitz, and Timothy syndromes (Persico and Napolioni, 2013).

Over time, genetic studies have led to a wide-genome approach, looking at both population studies and monogenic disorders

1.1.4.1 Array-CGH

Array-CGH analysis, due to its high detection rate (20–25%) in identifying causative genomic rearrangements (microdeletions/microduplications named copy number variations), is now recommended by Italian and International guidelines as a first-tier clinical diagnostic test for idiopathic intellectual disability and congenital malformations (Beaudet 2013; Italian Society of Human Genetics http://www.sigu.net; Miller et al. 2010; Al-Mamari et al. 2015). CNVs causing syndromic diseases are described in 8–21% of individuals with ASD and they are more frequent in severe phenotypes (Beaudet 2013; Jeste and Geschwind 2014; Schaefer et al. 2013; Miller, et al., 2010, Vicari et al., 2019).

It is estimated that CNVs can contribute to the genetic susceptibility to ASD in approximately 10% of subjects and provide a diagnostic rate of 5-10%. At present, there is a limited understanding of the role that these variants play in disease causation, particularly those inherited from seemingly unaffected parents. Overall, the effort to identify the genetic background that causes or contributes to ASD symptoms, could be hampered by studies on non-homogeneous patients, such as studies involving both complex and essential ASD.

However, our ability to identify causative genetic risk factors in a single individual remains scarce.

1.1.4.2 Next Generation Sequencing

Next Generation Sequencing (NGS) is a term used to describe a set of sequencing technologies that have allowed clinicians to examine much large sample of genetic material at a relatively lower cost than traditional techniques, such as Sanger sequencing. NGS was typically used to identify "single nucleotide variants" (SNVs), as well as small insertions or deletions in candidate genes. However, NGS can also be used to identify both "Copy number Variants" (CNVs) and balanced chromosomal rearrangements.

The NGS technique is typically divided into 3 categories, which vary in terms of coverage (i.e. the measurement of the target sequence, ranging from one to a few genes to the entire genome) and genetic resolution. In general, the smaller the genetic coverage, the higher the resolution is. It should also be noted that the more the target sequence increases in size, the greater is the risk of false positives and negatives.

1.1.4.2.1 Target gene panels

Genetic panels usually analyze 50-100 genes that have been shown to be related to a particular pathology. As mentioned before, this method, which of the three has the

lowest coverage, offers the highest resolution. The use of this method has the advantage of a high sensitivity but does not allow the identification of mutations in genes outside the panel, which can represent a disadvantage in a genetically heterogeneous pathology such as autism.

1.1.4.2.2 Whole exome sequencing

The term exome is a term derived from the word Genome and indicates the set of all the exons present in the genome, or of all the subsequences of DNA that can encode proteins. Although the exome represents only 1% of the human genome, it is estimated to contain the 85% of all disease-mutations and single nucleotide polymorphisms (SNPs) that appear in coding regions are the most common cause for Mendelian diseases (single-gene-dependent diseases). The disadvantage is that it does not identify any mutations present in non-coding regions. (Choi et al, 2009) (Horner et al, 2010)

1.1.4.2.3 Whole genome sequencing

This approach allows to cover the entire genome, allowing the sequencing of both the coding and non-coding regions, thus allowing to identify single nucleotide changes or small deletions or duplications.

Since 2011, a large number of studies have already been published using these techniques in ASD populations, with the identification of rare variants in autism susceptibility genes already described or newly identified. (O'Roak, et al., 2012) (Sanders, et al., 2012 and 2015) (Neale, et al., 2012), (Iossifov, et al., 2012 and 2014), De Rubeis et al. (2014), Sanders et al. (2015), Yuen et al. (2017), Ruzzo et al. (2019), Doan et al. (2019), Satterstrom et al. (2020) These studies have led to an exponential increase in the number of genes potentially related to autism. Moreover, the results of these studies demonstrate an extreme genetic heterogeneity of the disorder, as it

is evident from the findings, that it seems to be a minimal overlap between the candidate genes reported in the various studies.

1.1.4.3 Genetic architecture

The genetic architecture of ASD is extremely complex and still challenging, although there are putative models (Chaste et al, 2017).

Kleijer and colleagues in 2017, and more recently Pizzo et al (2019,) took an interesting overview in which underline that the susceptibility to ASD can be different from one individual to another with a combination of rare deleterious variants (low contribution) and many low-risk alleles (genetic background). Moreover, a relatively recent review tries to simplify this complexity identifying three major components of genetic risk, including polygenic variation, rare variants, and *de novo* mutations (Iakoucheva et al 2019).

According to this hypothesis, genetic background can influence the impact of rare inherited or *de novo* variants modulating the phenotypic heterogeneity observed in ASD patients.

As ASD is recognized as a spectrum of conditions, at one side of the spectrum seems to stand monogenic disorders, in which a copy number variation or a single gene mutation gives a major contribution to genetic risk, considering that severe mutations in any of a large set of genes are sufficient to cause the disorder: this model is called "heterogeneity model". At the other side there is the so called "infinitesimal model", that emphasizes the major role played by common variation in the documented high heritability of ASD; it is also known as polygenic risk model, that is the results of many common risk alleles that have each a small effect on the phenotype (Grove et al, 2019).

Notably, while heterogeneity and additive models are fundamentally different, they can share key elements.

In the middle, we identify a hybrid model, that possibly cover the most part or the total of individuals. It asserts that common and rare variations combine in some way, perhaps additively, to confer liability, defining a spectrum of conditions of complex genetic inheritance, that goes from cases in which risk is identified in multiple rare variants (for example a rare gene variant and a large duplication), and cases in which risk is attributable to a *de novo* gene mutation and to an increased load of polygenic risk inherited from both parents. (Weiner et al 2017).

In a population, common variation probably plays the dominant role in liability, whereas a rare mutation can make the largest contribution to liability for an individual who carries it (Gaugler et al, 2014).

Still, our current understanding of the genetic architecture of ASD is unsatisfactory, especially regarding how common and rare variations jointly confer risk. This architecture is important because it has clinical consequences; for example, it could require more nuanced evaluation of recurrence risk. Establishing the exact nature of the interplay between common and rare risk variations will be challenging, however, because of the multiplicity of putative models that could fit the current data.

Some studies tried to find the contribution of high and low risk variants to the pathogenesis of the disease.

Girirajan and colleagues (2010) studied patients with 16p12.1 microdeletions and found that the patients with a more severe phenotype and outcome had another CNV additively to the pathogenetic one. They concluded that most of these deletions were inherited from parents and that neuropsychiatric manifestations or "traits" were significantly more frequent in carrier parents compared to non-carrier ones.

This assumption is stated also by a recent work by Oetjens and colleagues (2019). They show that, in subjects carriers of a mutation in one of 11 selected rare genetic conditions, the quantitative traits associated to these disorders vary substantially as

a function of the common genetic variation they are carrier of; for this reason, they assume that these rare and common variants could act additively to the phenotype. Moreover, they note that it would be hard to discriminate additive from non-additive models even for this last case without studying larger samples.

Another large NGS study reaches similar conclusions, as the authors hypothesize that common genetic variants, in or near ASD associated genes, affects the risk of autism, although the sample sizes do not have the power to detect the convergence of the two (Satterstrom et al, 2020).

In the context of ASD and its binary diagnosis, distinguishing heterogeneity and additive models will be even more challenging (Klei et al, 2021).

The link between clinical and genetic aspects is not only directly causative, but it implies the role of the product genes in pathways related to developmental processes in early development (such as neural migration, synaptogenesis, micro and macrostructure of the brain).

Many genes associated to high risk for ASD encode for proteins that are involved in crucial role for synaptic function in the brain; these include proteins of the *SHANK* family proteins, that are postsynaptic proteins involved in scaffolding (i.e. *SHANK 2/3*), neurexins (i.e., *NRXN1*) and neuroligins (i.e., *NLGN2, NLGN4X)*, both part of the cell adhesion family.

Based on in vitro and in vivo models, many ASD-associated genes were found to be involved in pathways responsible for protein synthesis and degradation, chromatin remodeling, and synaptic function, converging in their role in synaptic homeostasis and synaptic plasticity. Therefore, ASD is also classified as a synaptopathy (Guang et al, 2018). In the synapses, two signaling pathways seem to be fundamental for ASD pathogenesis. The mTOR/PI3K pathways seems to be strongly associated to

syndromic ASD and the *NRXN-NLGN-SHANK* pathway. Together, these pathways are key regulators of synaptogenesis.

However, they are mainly located in excitatory synapses, and this can lead to an unbalance between excitatory and inhibitory pathways.

It has been hypothesized that many genetic and non-genetic risk factors converge at a synaptic level (Grabucker et al, 2013).

1.1.5 Beyond genetics

Moreover, the complexity rises if we consider the fact that not only genetic aspects seem to be involved in the determination of the phenotype in ASD.

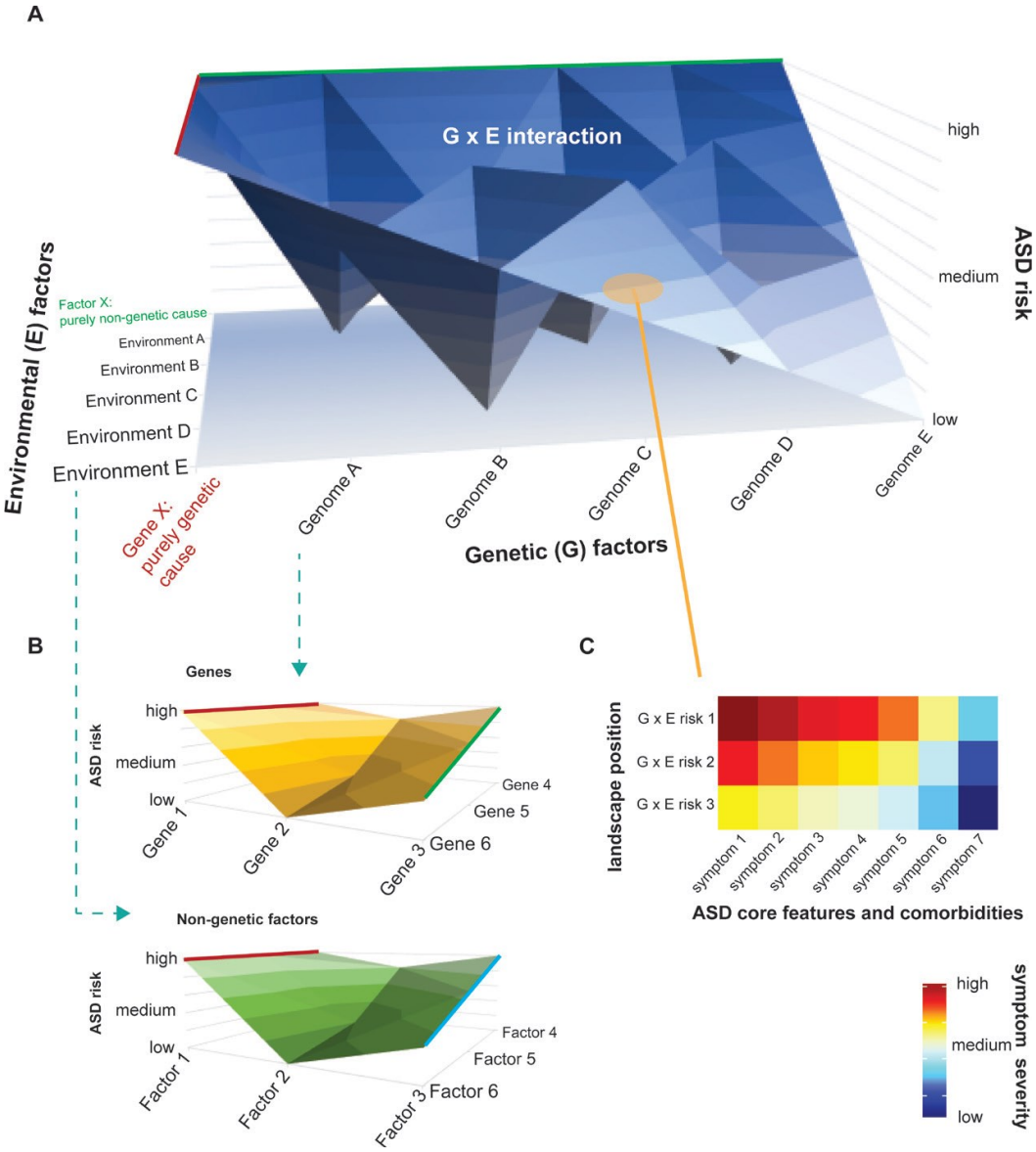
Several research indagated and identified environmental risk factors for autism, as advanced parental age and birth trauma, particularly if due to proxies of hypoxia, maternal obesity, a short interval between pregnancies, gestational diabetes mellitus and valproate use during pregnancy. (Modabbernia et al, 2017).

They derive from studies on large populations through statistical analysis, and they cannot be considered directly causal, but reactive, independent, or contributory for autism.

Moreover, many studies are approaching the disorder from different points of view, looking at autoimmunity and immunity, epigenetics, environmental factors.

What is known is that the etiology of ASD is likely to be multifactorial not only from a genetic point of view, but both genetic and nongenetic factors playing a role.

Figure 3 - Model for the etiology of ASD (Sauer et al, 2020)



A clarifying representation is proposed by Sauer and colleagues (2021) (Figure 3). They try to represent the complex hypothesis of gene-environment interaction that could contribute to ASD etiopathogenesis, starting from the assumption that ASD could be caused by genetic or environmental factors or a combination of these.

In some cases, ASD diagnosis is made in subjects with a known genetic abnormality that, alone, is considered causative for behavioral ASD diagnosis. (Figure 3, panel A, red line). In the same way, exposure to a certain environment or environmental factor should cause ASD (hypothetically) in an independent way from the genetic background (Figure 3, panel A, green line). This hypothetically models leads to two opposite side of a spectrum, but, as said about genetics, each individual undergoes to both genetic asset and environmental factors; in this way the genetic setup of an individual, in combination with the exposure to environmental factors, will determine the risk for ASD.

As represented in Figure 3 we can figure out the genetic factors, with rare variants of high impact (red line, upper panel), low risk genes that can add or diminish risk (green line, upper panel) and the environmental factors with their relatively risk (lower panel, part B). Putting together, these aspects, alone or together, can impact on an hypothetically threshold, that, surpassed, will cause the disorder, and can determine its heterogeneous phenotype (part C).

This model is evidently a simplification, because it does not take in account many other aspects that reasonably impact on ASD pathogenesis, such as immunological, neurophysiological and microstructural aspects.

1.2 Background

Since the etiopathogenesis of ASD is thought to be multifactorial and multigenic, we initially chose a wide-genome approach using array CGH analysis, to look for occurrence of rare variants or recurrent CNVs that could be related to ASD. The aim of our first study was to evaluate the presence of CNVs and to determine the diagnostic rate of array-CGH in a group of 209 patients with complex and essential ASD, and to investigate the possible relationship between genetic findings and clinical variables, i.e. familiarity for neuropsychiatric disorders, and

cognitive/behavioral characteristics (intelligence level, ASD severity, presence/absence of language) (Annunziata et al 2021).

We accurately assessed subjects classifying them in complex (47/209) and essential (162/209), and performed array comparative genomic hybridization, to identify causative and recurrent Copy Number Variants (CNVs) .

We found 117 CNVs in 75 patients, 10 classified as pathogenic, that is a diagnostic rate of 5,2%, less than expected according to the literature. In particular, we found pathogenic CNVs in 12,8% (6/47) of complex subjects and only in 3,1% (5/162) in essential ones. Moreover, the subjects with complex ASD have higher frequency of causative CNVs than subjects with essential ASD as only few previous studies have already found (Toriello, 2012; Jacquemont et al., 2006; Lovrečić et al., 2018).

In literature we found a higher rate of diagnostic array-CGH in mixed or essential ASD population, estimated in about 10%. Even if a recent study on subjects with essential ASD confirms pathogenic CNVs in 9% (12/133) of individuals (Napoli et al., 2018), our results seem to be more in line with that of Pinto and colleagues (2014). They studied 2446 families, selecting essential ASD, and found pathogenic events in 2.8% of affected subjects, in accordance with the diagnostic rate of our subjects with essential ASD.

We found lower percentage of *de novo* events in the whole sample (1,9%), even if it is about 1,2% in subjects with essential ASD but 4,2% (2/47) in complex ASD, that is more similar to that reported by other studies (Sebat et al., 2007; Jacquemont et al., 2006; Pinto et al., 2014). We must consider that only 65% of patients (49/75) have been studied for CNVs segregation and this can be considered a bias. Considering only CNVs tested for segregation, we found a *de novo* rate similar to that described in literature (4,8%). There is to notice, thereby, that populations of other studies are often mixed because the subjects were not well characterized phenotypically; it is

then possible to hypothesize that the accurate selection of our patients reduces the rate of *de novo* CNVs in patients with essential ASD.

Cognitive and verbal abilities, and severity of autistic symptoms were not related to genetics findings.

Moreover, the presence of more than one CNVs was found in 25 patients, sometimes inherited from both parents. Some studies suggest that two or more CNVs can interact and predispose carriers to neuropsychiatric disorders (Stankiewicz & Lupski, 2010). Other studies have found that an increase in CNVs number could be potentially related to neurodevelopmental disorders in probands belonging to families with a positive history of neuropsychiatric disorders, supporting the hypothesis of a possible increased susceptibility to ASD and related phenotypes (Bremer et. al., 2011).

In our sample all patients were evaluated through a deep familial anamnesis with particular attention to neuropsychiatric disorders, including up to third degree-relatives. We found positive neuropsychiatric familiar history in 5 of the 11 patients with normal IQ, but the association with the number of CNVs was not statistically significant.

Looking at the number of CNVs, we found that 12 of the 25 patients with more than one CNV have a positive family history for neurodevelopmental or psychiatric disorders.

We can therefore hypothesize that if, on one hand, there could be a ASD subgroup with a greater predisposition to de novo events (for example related to parental age), on the other hand there could be another group with an increased genetic susceptibility due to inherited CNVs, which leads to the development of the phenotype through the action of unknown factors (environmental or genetic). This data emphasizes the importance of the role, for pathogenic purposes, not only of de novo variants, but also of inherited CNVs.

Our findings confirmed the need for a genetic model of ASD with a high level of complexity and the need for instruments and criteria to cluster ASD in endophenotypes. This must consider not only the overall incidence of the disorder and the difference in prevalence between males and females, but also the presence of different classes of risk in affected individuals and families, with the possibility of phenotypic expression mediated by incomplete penetrance mechanisms and variable expressivity. It must also be considered that genetic alterations significantly associated with ASD are also present in other neurodevelopmental and psychiatric disorders (Ronemus et al., 2014).

Our previous work underlined the diagnostic value of array-CGH in so-called complex autism, and its positive predictive factor in cases with malformation and peculiar facial characteristics.

We then decided to deepen our study following the genetic asset of essential autism, and of those cases in which all genetic and metabolic exams were negative.
As we underlined the role of array-CGH in complex autism, we therefore decide to deepen our genetic study in patients with essential ASD and negative array-CGH.

1.3 Aim of the study

The aim of the present study is the genetic analysis with a targeted NGS gene panel of a group of selected ASD patients who tested negative to array CGH testing, without facial peculiar characteristics and/or different kind of malformations, and without major anomalies at MRI and EEG screening. The panel we designed contains 120 selected genes. Our goals are to evaluate the presence of rare genetic variants inherited or *de novo* in patients and in their parents and to better understand their role in ASD phenotype.

2 Methods

2.1 Subjects

We recruited 86 patients with Autism Spectrum Disorder (73 males, 13 females) who referred to Neurodevelopment Disorders Unit of C. Besta Institute between January 2017 and July 2019.

Diagnosis of Autism Spectrum Disorder followed diagnostic criteria of DSM-5 and was confirmed by clinical examination consisted of an accurate anamnesis, Autism Diagnostic Interview-Revised, ADI-R (Lord et al, 1994) and the Autism Diagnostic Observation Schedule – II edition, ADOS2 (Lord et al., 2000).

Cognitive functioning was assessed using the Wechsler Intelligence Scales (Weschler 2003), according to their age, and the Griffiths Mental Developmental Scale for children with chronological or mental age under 4 years (Griffiths 1996) (Green et al 2016), considering the correlation between Wechsler Preschool Scale Full IQ and General quotient obtained by Griffiths Scales (Sutcliffe et al, 2010).

Developmental delay or intellectual disability were defined by, respectively, a GQ or a QI lower than 70, and we moreover classified ID/DD in grade as follows: mild (IQ 50-70), moderate (35-50), severe (20-35).

All patients underwent to an accurate clinical evaluation and an anamnestic record, with particular attention to familial history. We ask for familial history of neurodevelopmental disorders or neuropsychiatric conditions, up to second-degree-relatives.

All the patients were evaluated by a pediatric neurologist, a clinical geneticist, and a child neuropsychologist and underwent also to the following instrumental screening:

- brain magnetic resonance imaging (MRI) 1.5 Tesla without contrast enhancement (with sagittal, transverse and coronal sequences, and FLAIR sequences of the whole brain and cerebellum);
- electroencephalogram (EEG);
- plasma amino acid assay, using liquid chromatography tandem mass spectrometry (ESI/MS/MS); urinary organic acid assay, using gas chromatography mass spectrometry;
- standard karyotyping on peripheral blood, by means of lymphocyte culture; QFQ banding, resolution 550 bands (number of metaphases analyzed: 16);
- molecular analysis for fragile X syndrome, using Southern blot.
- Array Comparative Genomic Hybridization

We excluded patients with:(1) preterm birth or known pregnancy complications or perinatal injuries history; (2) major neuroradiologic alterations (i.e. cortical dysplasia), (3) epileptic syndromes (i.e. West syndrome); (4) known infectious or metabolic dis-eases; (5) major facial and somatic peculiar characteristics; (6) major limb's or visceral malformations; (7) presence of undergrowth or overgrowth syndromes (8) genetic diseases (high-resolution karyotype, DNA analysis of Fragile-X syndrome) or positive genetic testing, including the presence of any Copy Number Variants in aCGH testing.

All the examinations were performed with written informed consent of the subjects' parents. The study was approved by the Ethics Committee of Fondazione IRCCS Istituto Neurologico C. Besta.

2.2 Genetic analysis

Genomic DNA was extracted from the whole blood of all the patients enrolled in the study and, when possible, of their parents.

DNA samples were analyzed by targeted resequencing using customized gene panels (Nextera Rapid Capture Custom Enrichment, Illumina) based on a systematic literature review. In detail, we examined: (i) targeted genes panel available for sale (TruSight Autism Target Genes and TruSight Inherited Disease Target Genes, Illumi-na), (ii) main genes implicated in ID and ASD reported in literature (Redin C et al. 2014, Cukier et al. 2014, Iossifov et al. 2012, Liu X. et al. 2014), (iii) and SFARI gene database (the most important and complete Autism variants data-base) (Table 2), that lists about 1000 genes associated with ASD.

A total of 120 genes was selected, custom probes were designed focusing on genes coding sequences(CDS) with +/- 20 bp intronic flanking region to include splicing regulatory elements.

Table 2- List of 120 genes included in NGS panel

AGAP1	DHCR7	IQSEC2	PCDH19	SLIT3
ANK2	DLGAP2	JARID2	PCDH9	SMG6
ANKRD11	DMD	KATNAL2	PHF6	SNRPN
AP1S2	DOCK4	KCNMA1	PHF8	SOX5
ARX	DPP10	KCTD13	POGZ	SPAST
ASH1L	DST	KDM5C	PRICKLE1	STXBP5
ASTN2	DYRK1A	KDM6B	PTCHD1	ST7
ATRX	EHMT1	KIRREL3	PTEN	SYNGAP1
AUTS2	EN2	LAMC3	PTPN11	SYN1
AVPR1A	FAT1	MBD5	RAB39B	TBR1
BDNF	FGD1	MECP2	RAI1	TCF4
BRAF	FMR1	MED12	RBFOX1	TRIMM33
CACNA1A	FOXG1	MED13L	RELN	TSC1
CACNA1C	FOXP1	MEF2C	RIMS1	TSC2
CACNA1H	FOXP2	MET	RPL10	UBE3A
CADPS2	GABRB3	NF1	SATB2	VPS13B

CASK	GNA14	NIPBL	SCN1A	WNT2
CDKL5	GRIN2B	NLGN3	SCN2A	YWHAE
CEP290	GRIN2A	NLGN4X	SHANK2	ZEB2
CHD8	GRM5	NRXN1	SHANK3	ZNF804A
CNTNAP2	GRPR	NRXN3	SLC16A2	
CNTN4	HCFC1	NSD1	SLC6A3	
CREBBP	HOXA1	NTNG1	SLC6A4	
CSMD1	IL1RAPL1	OPHN1	SLC9A6	
CUL4B	IMMP2L	OXTR	SLC9A9	

Libraries enrichment was performed according to Nextera Rapid Capture Enrichment protocol (Illumina), generated libraries were then loaded and sequenced on MiSeq platform (Illumina).

The generated reads were aligned to human genome assembly hg19 (Human Feb. 2009 GRCh37) using BWA (v0.7.9a) and variants call was performed using GATK (v1.6-23). The variants identified were annotated and filtered (Variant-Studio3.0, Illumina), focusing on rare variants with minimum allele frequency <1% in gnomAD database [www.gnomad.broadinstitute.org]). We classified all these rare variants, that have overcome quality filters, in five additional subcategories (Table 3).

Table 3- Frequency subcategories of rare variants

Group	Allele frequency (AF)
***	(AF)<1/10000 (0,01%)
**	1/10000 (0,01%)<AF<1/5000 (0,02%)
*	1/5000 (0,02%)<AF<1/1000 (0,1%)
-	1/1000 (0,1%)<AF<1/500 (0,2%)
f	1/500 (0,2%)<AF<1/100 (1%)

All variants found were validated by Sanger sequencing, in addition, for trios with available parents' DNA, variants segregation was investigated.

2.3 Statistical Analysis

Statistical analyses were employed by SPSS Statistics 20 software (IBM Corp., 2011). Pearson correlation (r) has been used to evaluate the relationship between total variants number and cognitive and behavioral data (respectively, Total IQ and ADI-R and ADOS scores).

Moreover, we carried out independent t-test to evaluate if number of variants per subject is different on the basis of the familial history for neuropsychiatric disorders. All the statistical analyses were two-tailed and p-values of .05 or lower were considered significant.

We compared mutational frequency of the 6 most mutated gene in our cohort and in GnomAD population using a binomial test. To define GnomAD population frequency we calculated for each gene the number of rare variants (present in less than 1% of the population) in a variable number of alleles, considering coverage data. Synonymous and intronic variants were excluded from the counting.

We finally used STRING (https://www.string-db.org/), that is a database used to build predicted and well-known PPI networks. The interactions in STRING are mainly derived from automated text-mining and databases of previous knowledge, among other resources database of known and predicted protein-protein interactions.

For each patient with more than 5 variants we search the presence of significant network enrichment between the genes in which we found variants. We further look at the type of enrichment results.

3. Results

3.1 Clinical evaluation

We recruited 86 patients with a diagnosis of ASD. Out of the 86 patients recruited, 73 were males and 13 females, with a male to female ratio 5,6:1. Age at the first evaluation ranged from 20 months to 177 months (mean 53 months, SD 29).

Cognitive assessment was attempted in 83 of 86 enrolled subjects and was successfully completed in 71 patients: 4 had a normal IQ (above 85), 5 were borderline (75-85), 32 had a mild intellectual disability (55-75), 20 moderate intellectual disability (40-55), 10 severe intellectual disability (25-40); the remaining 13 children were not testable because of lack of collaboration due to behavioral problems (opposition, hyperactivity, stereotyped use of the material); these patients had all an history of psychomotor delay and impairment in adaptive behavior life skills.

ADOS-2 and ADI-R were administered to those patients who fulfilled the inclusion criteria (for ADI-R mental age above 24 months, for ADOS-2 non-verbal mental age above 18 months for Module 1 and above 12 months for Toddler Module).

We administer ADOS-2 in 71 children, in 5 children Toddler Module, in 52 Module 1, in 6 Module 2 and in 8 Module 3. We administered ADI-R in 67 patients. A summary of neuropsychological data is presented in Table 4.

We recruited mainly "simplex" families (with no other ASD case in first-degree relatives), only one multiplex family was included, with two siblings (C17/76 and LDM568), a male and a female.

As for geographical provenience, in our sample 58 have both Italian parents, 6 have only one Italian parents (the other parent came from Eastern Europe in 4 subject and from South America in the other 2); as for the rest, 11 came from Eastern Europe, 6 came from Africa, 5 came from Asia.

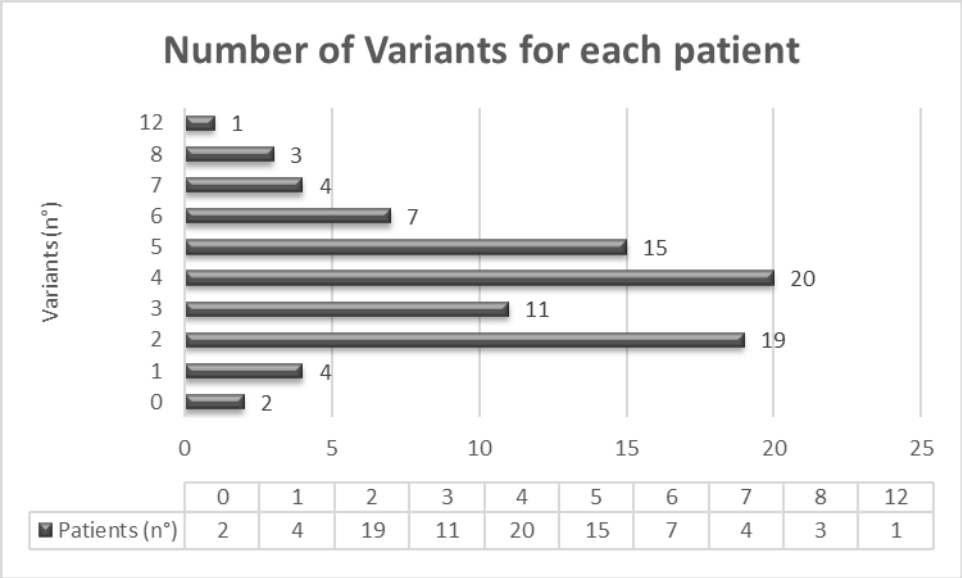
Table 4- Clinical data

	Mean (Standard Deviation)
GQ or TIQ (N 71)	57.2 (15.9)
ADI (N 67)	
Social	1.38 (0.4)
Communication	1.54 (0.4)
RR behaviors	1.12 (0.5)
ADOS (N 71)	
Social affect	1.5 (0.36)
RR behaviors	1.25 (0.5)
Comparison score	7.7 (1.7)

3.2 Gene panel

We found 336 variants in 84 of 86 patients (72 males, 12 females) analyzed by NGS (which fall into 87 of 120 genes): only two patients (H16/18 and G16/59) have no significant variants (Table 5 and Table 1Table 16 in the Appendix). Moreover, only 4 patients showed a unique variant, while in the rest of the cohort (80 out of 86 patients) we found more than one variant for each patient (see Figure 4).

Figure 4 - Number of variants for each patient



The variants taken into consideration have a GnomAD frequency <1%. Those with a greater frequency have been discarded.

We found 321 missense, 4 synonymous predicted to alter normal splicing, and 11 indels, all in heterozygous state, except one missense variant in *MET* gene, carried by both alleles. Four variants in *DMD* gene, 3 in *MED12* gene and single variant in *CDKL5*, *HCFC1*, *NLGN3*, *PCDH19*, *PTCHD1* and *SLC16A2*, located on Chromosome X, are present in male patients in hemizygous state.

Table 5 - Summary of all variants for each patient, with family segregation, frequency and in vitro prediction information

Patient	Total Variants	Genes	Paternal variants	Maternal variants	*De Novo* variants	Variants already associated with ASD	Variants very rare (***, **)	Variants in most mutated genes	Variants found more than one	Variants with at least one deleterious prediction
1 (H16/18)	0		0	0	0	0	0	0	0	0
2 (E10/24)	4	*MED12, CACNA1C, DLGAP2, DHRC7*	U	U	U	0	3	0	0	3(***,***,**)
3 (O12/11)	5	*CNTNAP2, NRXN1, SOX5, ANKRD11, CDKL5*	2 (***,f)	3 (***,**,f)	0	1 (M, f)	3 (P,M,M)	1 (ANKRD11, P, f)	2 (M,f)(P,f)	1 (M,f)
4 (C14/16)	2	*ASH1L, PS13B*	U	U	U	0	2	1 (VPS13B,***)	0	1 (***)
5 (C.P.P)	5	*SLIT3, DLGAP2, CACNA1C, RAI1, MED13L*	2 (*,***)	3 (f,*,***)	0	0	2 (P,M)	0	1 (f,M)	3 (P,*)(M,*)(P,***)
6 (C.L.)	7	*MBD5, TSC1, FAT1, SLIT3, SHANK3, SCN2A, ZEB2*	U	U	U	1 (*)	3	1 (FAT1,***)	2 (f,f)	3 (*)(***)(f)
7 (A14/06)	6	*NRXN1, NRXN3, ZNF804A, VPS13B, RELN, RAI1*	3 (*,f,f)	3 (***,***,f)	0	1 (P,f)	2 (M,M)	1 (VPS13B,M,f)	0	4 (M,***)(M,***)(P,*)(P,f)
8 (D.F.M.)	1	*JARID2*	1(**)	0	0	0	1 (P)	0	0	0
9 (E13/58)	2	*GRIN2B, PCDH9*	U	U	U	0	2	0	0	0
10 (A14/55)	2	*ANKRD11, NF1*	0	0	2 (-, ***)	0	1 (*D.N.*)	1 (ANKRD11, D.N., -)	1 (D.N.,-)	1 (*D.N.*, -)
11 (G14/62)	7	*DST, DLGAP2, RAI1, ZNF804A, ZNF804A, CEP290, SYNGAP1*	2 (***,***)	5 (***,***,*,*,f)	0	0	4 (P,P,M,M)	0	1 (M,f)	4 (P,***)(M,***)(M,*)(M.f)
12 (G.F.M.)	2	*MED12, DST*	1 (*)	1 (***)	0	0	1 (M)	0	0	1 (M,***)
13 (IB1A)	5	*KIRREL3, KDM6B, DMD, DHCR7, RAI1*	4 (***,***,*,***)	1 (**)	0	0	4 (P,P,P,M)	0	0	4 (P,***)(M,**)(P,***)(P,***)
14 (IB17A)	2	*NSD1, SCN2A*	U	1 (f)	U	0	1	0	1 (M,f)	2 (U,***)(M,f)
15 (A15/18)	9	*ZNF804A, VPS13B, VPS13B, SHANK3, ASH1L, SPAST, FAT1, AUTS2*	U	U	U	0	6	3(VPS13B,***)(VPS13B,***) (FAT1,**)	0	3 (***)(***)(**)
16 (E11/61)	4	*FAT1, GRIN2A, GNA14, GNA14*	U	U	U	0	0	1 (FAT1,-)	0	3 (-)(-)(f)
17 (F11/04)	3	*CACNA1H, DMD, GNA14*	2 (***,f)	1 (***)	0	0	2 (P,M)	1 (CACNA1H,P,***)	0	3 (P,***)(M,***)(P,f)

Patient	Total Variants	Genes	Paternal variants	Maternal variants	*De Novo* variants	Variants already associated with ASD	Variants very rare (***, **)	Variants in most mutated genes	Variants found more than one	Variants with at least one deleterious prediction
18 (F12/67)	2	*FAT1, FAT 1*	2 (***,*)	0	0	0	1 (P)	2 (FAT1,P,***)(FAT1,P,*)	0	2 (P,***)(P,*)
19 (D15/81)	4	*MET, SLC6A4, VPS13B, CACNA1H*	3 (f,f,f)	1 (***)	0	1 (P,f)	1 (M)	2 (VPS13B,P,f) (CACNA1H,P,f)	3 (P,f)(P,f)(P,f)	1 (P,f)
20 (F14/70)	5 (1 Homo)	*ASH1L, NSD1, MET, MED13L, POGZ*	2 (***,***)	4 (***,***,***,-)	0	0	4 (P, P/M,M,M)	0	1(M,***)	3 (P,***)(M,***)(P/M,***)
21 (M.L.B.)	1	*ANK2*	U	U	U	0	1	1(ANK2,***)	0	0
22 (N13/03)	4	*TSC2, CADPS2, DLGAP2, CEP290*	2 (***,f)	2 (*,f)	0	0	1 (P)	0	3 (M,f)(M,*)(P,f)	3 (P,***)(M,*)(M,f)
23 (L12/26)	5	*AGAP1, LAMC3, NRXN1, ZNF804A, CACNA1H*	4 (***,*,-,f)	1 (***)	0	0	2 (P,M)	1 (CACNA1H,P,-)	0	3 (P,***)(M,***)(P,f)
24 (B12/69)	4	*NSD1, ANK2, CSMD1, ASTN2*	1(***)	3 (**,-,f)	0	0	2 (P,M)	2 (ANK2,M,-)(CSMD1,M,f)	1 (P,***)	3 (P,***)(M,-)(M,**)
25 (D10/14)	3	*CREBBP, VPS13B, VPS13B*	U	U	U	1 (f)	0	2 (VPS13B,-)(VPS13B,*)	0	2 (f)(-)
26 (C11/79)	4	*ANK2, CACNA1H, TSC2, DMD*	3 (***,***,***)	1(***)	0	0	4 (P,P,P,M)	2 (ANK2,P,***)(CACNA1H,P,***)	0	1 (P,***)
27 (H11/97)	2	*RIMS1, FAT1*	U	U	U	0	1	1 (FAT1,f)	1 (f)	1 (f)
28 (C15/87)	5	*EHMT1, FAT1, DLGAP2, CSMD1, NLGN3*	1 (*)	4 (***,*,*,f)	0	0	1(M)	2 (FAT1,P,*)(CSMD1,M,f)	0	5 (M,***)(P,*)(M,*)(M,*)(M,f)
29 (C14/83)	4	*SLC9A9, DST, VPS13B, ANKRD11*	U	U	U	0	2	2 (VPS13B,f)(ANKRD11,*)	0	3 (***)(*)(f)
30 (M14/99)	2	*FAT1, EHMT1*	U	U	U	0	1	1 (FAT1,f)	1 (f)	2 (**)(f)
31 (L14/95)	5	*SLIT3, MBD5, ANK2, VPS13B, KDM6B*	0	4 (*,**,f,f)	1 (f)	1 (*D.N.*,f)	1 (M)	2 (ANK2,M,*)(VPS13B,M,f)	1 (M,f)	2 (M,**)(M,f)
32 (s.s.73499)	1	*FAT1*	1 (f)	0	0	0	0	1 (FAT1,P,f)	1 (P,f)	1 (P,f)
33 (r.g.74126)	5	*FAT1, ZEB2, CSMD1, AVPR1A, RAI1*	2 (f,-)	3 (*,f,f)	0	1 (M, f)	0	2 (FAT1,M,f)(CSMD1,M,f)	4 (P,f)(P,-)(M,f)(M,f)	4 (M,f)(M,f)(M,*)(P,f)
34 (a.d.81815)	5	*FAT1, CSMD1, ANKRD11, ZEB2, RAI1*	2 (***,f)	3 (***,***,-)	0	0	3 (P,M,M)	3 (FAT1,M,***)(CSMD1,P,***)(ANKRD11,M,***)	2 (M,-)(P,f)	2 (M,***)(P,f)
35 (g.w.g.01350)	2	*FOXP2, CSMD1*	U	U	U	0	2	1 (CSMD1,***)	0	2 (***)(***)

Patient	Total Variants	Genes	Paternal variants	Maternal variants	*De Novo* variants	Variants already associated with ASD	Variants very rare (***, **)	Variants in most mutated genes	Variants found more than one	Variants with at least one deleterious prediction
36 (p.a.59939)	4	*ANK2, AGAP1, VPS13B, CACNA1A*	2 (**,f)	2 (**,1)	0	0	2 (P,M)	2 (ANK2,P,**)(VPS13B,M,*)	0	3 (P,**)(M,**)(M,*)
37 (o.f.76867)	2	*ANK2, ST7*	2 (***,f)	0	0	0	1 (P)	1 (ANK2,P,***)	0	0
38 (H.S.M.)	4	*TSC1, LAMC3, ANKRD11, KATNAL2*	2 (*,-)	2 (**,*)	0	1 (M,*)	1 (M)	1 (ANKRD11,P,*)	1(M,f)	1 (P,-)
39 (LDM160)	4	*VPS13B, SHANK3, RELN, DMD*	1 (***)	3 (***,***,**)	0	0	4 (P,M,M,M)	1 (VPS13B,m,***)	0	3 (M,***)(P,***)(M,***)
40 (LDM167)	4	*PTEN, CREBBP, FAT1, VPS13B*	2 (***,f)	1(f)	1 (***)	0	2 (P,*D.N.*)	2 (FAT1,P,f)(VPS13B,M,f)	1 (M,f)	3 (*D.N.*,***)(P,***)(P,f)
41 (H14/09 B.F.)	4	*MBD5, NSD1, DST, AUTS2*	2 (***,*)	2 (***,***)	0	0	3 (P,M,M)	0	0	4 (M,***)(M,***)(P,*)(P,***)
42 (P.A.-79782)	5	*RELN, VPS13B, GNA14, NRXN3, ANKRD11*	1 (***)	4 (***,***,***,*)	0	1 (M,*)	3 (P,M,M)	2 (VPS13B,M,*)(ANKRD11,M,-)	1 (M,-)	3 (M,***)(M,-)(P,***)
43 (C.M.L.-63035)	3	*VPS13B, ANKRD11, SLC64A*	3 (***,**,*)	0	0	0	2 (P.P)	2 (VPS13B,P,*)(ANKRD11,P,***)	1 (P,*)	0
44 (P.J.A.-84366)	6	*NTNG1, AGAP1, DST, TSC1, PTPN11, ATRX*	2 (***,-)	4 (***,***,**,*)	0	2 (M,*)(P,-)	4 (P,M,M,M)	0	1 (M,*)	3 (M,**)(P,***)(M,***)
45 (M.S.-64656)	12	*POGZ, SATB2, ANK2 (2), NIPLB, RELN, CSMD1, CEP290 (3), ANKRD11, RAI1*	6 (***,***,***,***,*,*)	6 (**,*,*,f,f,-)	0	1(M,f)	5 (P,P,P,P,M)	4 (ANK2,M,**)(ANK2,M,-)(CSMD1,P,***)(ANKRD11,M,*)	0	6 (P,**)(M,-)(P,***)(M,f)(P,***)(P,*)
46 (M.A.-50264)	3	*SYNGAP1, MED13L, CHD8*	2 (***,f)	1 (***)	0	0	2 (P,M)	0	0	0
47 (A.A.A.K.-86859)	4	*DST, CADPS2, MED13L, SMG6*	3 (***,*,*)	1 (*)	0	0	1 (P)	0	0	3 (P,*)(P,*)(P,***)
48 (C17/76)	5	*CNTN4, DLGAP2, EHMT1, PTEN, CACNA1H*	2 (***,**)	3 (***,***,f)	0	1 (P,**)	4 (P,P,M,M)	1 (CACNA1H,M,***)	3 (P,**)(M,***)(M,f)	1 (M,***)
49 (LDM568)	5	*DLGAP2, PTEN, CACNA1H, KATNAL2, MED12*	3 (***,**,f)	2 (***,***)	0	1 (P,**)	4 (P,P,M,M)	1 (CACNA1H,M,***)	3 (P,**)(P,f)(M,***)	2 (M,***)(M,***)
50 (D.G.M.-50685)	5	*SLIT3, RELN, CEP290, PCDH9, KDM6B*	3 (***,***,f)	2 (***,*)	0	1 (P,f)	3 (P,P,M)	0	1 (P,***)	4 (P,***)(M,*)(P,f)(M,***)
51 (D16/37)	2	*KDM6B, CACNA1A*	0	2 (***,f)	0	0	1 (M)	0	0	1 (M,f)

Patient	Total Variants	Genes	Paternal variants	Maternal variants	*De Novo* variants	Variants already associated with ASD	Variants very rare (***, **)	Variants in most mutated genes	Variants found more than one	Variants with at least one deleterious prediction
52 LDM565	2	*LAMC3, MED12*	1 (**)	1 (-)	0	0	1 (P)	0	1 (M,-)	1 (P,**)
53 (LDM632)	6	*SLIT3, GRIN2A, VPS13B, NSD1, DYRK1A, PTCHD1, BRAF*	2 (f,f)	4 (***,*,*,-)	0	0	1 (M)	1 (VPS13B,P,f)	1 (P,f)	2 (M,*)(P,f)
54 (VLDM695)	1	*BRAF*	1 (P,***)	0	0	0	1 (P)	0	0	0
55 (51432)	2	*POGZ, CEP290*	1 (***)	1 (***)	0	0	2 (P,M)	0	0	1 (P,***)
56 (73206)	8	*MBD5, SCN1A, JARID2, STXBP5, SHANK2, TSC2, ANKRD11, DMD*	2 (***,f)	6 (***,***,***,***,**,-)	0	1 (M,-)	6 (P,M,M,M,M,M)	1 (ANKRD11,P,f)	1 (P,f)	6 (M,***)(M,-)(M,***)(P,***)(M,***)(M,**)
57 (73400)	4	*SCN2A, RELN, CSMD1, KDM6B*	2 (***,f)	2 (***,***)	0	0	3 (P,M,M)	1 (CSMD1,P,***)	0	1 (P,f)
58 (75544)	6	*ASH1L, VPS13B, CACNA1C, CACNA1H, TSC2, ANKRD11*	2 (f,f)	4 (***,*,f,f)	0	0	1 (M)	3 (VPS13B,M,f)(CACNA1H,P,f)(ANKRD11,M,f)	1 (M,f)	3 (P,f)(M,f)(M,***)
59 (52984)	3	*MBD5, SCN1A, NF1*	3 (***,***,f)	0	0	1 (P,***)	2 (P,P)	0	1 (P,f)	0
60 (65706)	7	*FAT1, SLIT3, AUTS2, VPS13B, SHANK2, CACNA1H, ANKRD11*	5 (***,***,**,*,-)	2 (***,**)	0	0	5 (P,P,P,M,M)	4 (FAT1,P,-)(VPS13B,M,**)(CACNA1H,P,**)(ANKRD11,P,***)	0	5 (P,-)(P,***)(P,*)(M,***)(P,**)
61 (H16/06)	2	*VPS13B, NRXN3*	1 (***)	1 (-)	0	0	1 (P)	1 (VPS13B,M,-)	1 (P,***)	1 (P,***)
62 (E17/07)	3	*FAT1, DST, MED13L*	3 (***,f,-)	0	0	0	1 (P)	1 (FAT1,P,f)	0	1 (P,f)
63 (G16/59)	0	/	0	0	0	0	0	0	0	0
64 (G17/11)	5	*NRXN1, GNA14, CACNA1C, CACNA1H, CREBBP*	3 (***,**,-)	2 (***,***)	0	1 (P,-)	4 (P,P,M,M,)	1 (CACNA1H,P,-)	0	1 (P,***)
65 (75323)	4	*HOXA1, GABRB3, SMG6, SLC6A4*	2 (f,f)	2 (***,*)	0	1 (P,f)	1 (M)	0	1 (P,f)	2 (M,***)(P,f)
66 (LDM733)	6	*GRIN2A, LAMC3 (2), KDM6B, FAT1 (2)*	4 (*,f,f,-)	2 (**,-)	0	1 (P,f)	1 (M)	2 (FAT1,P,f)(FAT1,P,-)	1 (P,f)	3 (P,f)(P,f)(P,-)
67 (52724)	2	*VPS13B, GRM5*	1 (***)	1 (*)	0	1 (M,*)	1 (P)	1 (VPS13B,M,*)	0	1 (M,*)
68 (70102)	6	*MBD5, FAT1, STXBP5, VPS13B, NRXN3, TSC2*	4 (***,***,**,f)	2 (*,f)	0	1 (P,f)	3 (P,P,P)	2 (FAT1,P,f)(VPS13B,P,***)	1 (P,f)	5 (P,**)(P,f)(P,***)(P,***)(M,*)

Patient	Total Variants	Genes	Paternal variants	Maternal variants	*De Novo* variants	Variants already associated with ASD	Variants very rare (***, **)	Variants in most mutated genes	Variants found more than one	Variants with at least one deleterious prediction
69 (85256)	4	*CEP290, PCDH9, NRXN3, DMD*	0	4 (***,***,f,f)	0	0	2 (M,M)	0	1 (M,***)	3 (M,f)(M,***)(M,***)
70 (LDM763)	2	*SMG6, HCFC1*	1 (***)	1 (-)	0	0	1 (P)	0	0	1 (M,-)
71 (A17/97)	3	*ASH1L, MBD5, ASTN2*	3 (***,***,f)	0	0	0	2 (P,P)	0	1 (P,f)	2 (P,***)(P,***)
72 (79149)	3	*TRIM33, DLGAP2, CACNA1C*	2 (***,f)	1 (f)	0	0	1 (P)	0	1 (P,f)	1 (P,***)
73 (D17/73)	2	*ANK2, PCDH19*	1 (***)	1 (-)	0	0	1 (P)	1 (ANK2,P,***)	0	0
74 (E17/77)	3	*NSD1, DST, CREBBP*	1 (***)	2 (***,***)	0	0	3 (P,M,M)	0	0	2 (P,***)(M,***)
75 (F17/16)	2	*KCTD13, ANKRD11*	2 (***,***)	0	0	0	2 (P,P)	1 (ANKRD11,P,***)	0	1 (P,***)
76 (H16/64)	6	*IMMP2L, CADPS2, CSMD1, CHD8 (2), SLC16A2*	2 (***,-)	4 (***,*,*,*)	0	0	2 (P,M)	1 (CSMD1,M,***)	0	3 (M,*)(M,*)(M,***)
77 (D17/50)	4	*ASTN2, CHD8, NRXN3, ANKRD11*	2 (***,f)	2 (***,***)	0	1 (P,f)	3 (P,M,M)	1 (ANKRD11,M,***)	1 (M,***)	3 (P,f)(P,***)(M,***)
78 (E17/49)	4	*SCN1A, DST, ANKRD11 (2)*	2 (*,f)	2 (***,f)	0	0	1 (M)	2 (ANKRD11,P,f)(ANKRD11,M,f)	2 (P,f)(M,f)	0
79 (LDM1089)	3	*SCN1A, CADPS2, PCDH9*	1 (-)	2 (***,*)	0	1 (M,*)	1 (M)	0	1 (M,*)	2 (M,*)(M,***)
80 (D17/27)	8	*MBD5, FAT1 (2), SLIT3, NSD1, IMMP2L, CSMD1, NRXN3*	5 (***,***,*,f,f)	3 (***,***,f)	0	0	4 (P,P,M,M)	3 (FAT1,M,f)(FAT1,P,*)(CSMD1,P,***)	2 (P,f)(M,f)	2 (M,f)(P,***)
81 (F14/24)	3	*RELN, CHD8, DMD*	U	1 (*)	U	0	0	0	0	2 (*)(*)
82 (D17/19)	5	*NRXN1, SCNA2, CSMD1, VPS13B, CACNA1A*	2 (*,f)	3 (***,***,***)	0	1 (P,f)	3 (M,M,M)	2 (CSMD1,M,***)(VPS13B,P,*)	2 (P,*)(P,f)	2 (P,f)(M,***)
83 (D17/35)	4	*ANK2, LAMC3, BDNF, ANKRD11*	3 (**,f,-)	1 (***)	0	0	2 (P,M)	2 (ANK2,M,***)(ANKRD11,P,f)	1 (P,f)	2 (P,**)(P,-)
84 (70555)	7	*CSMD1, VPS13B (2), KCNMA1, CEP290, MED13L, CACNA1H*	3 (*,*,f)	4 (*,*,f,f)	0	0	0	4 (CSMD1,M,f)(VPS13B,M,*)(VPS13B,M,*)(CACNA1H,M,*)	3 (P,f)(M,f)(M,f)	5 (M,f)(M,*)(M,*)(P,f)(M,f)
85 (73664)	2	*VPS13B, CACNA1H*	1 (f)	1(-)	0	0	0	2 (VPS13B,P,f)(CACNA1H,M,-)	1 (P,f)	0
86 (80147)	4	*RELN, ST7, WNT2, VPS13B*	3 (***,f,f)	1 (-)	0	1 (P,f)	1 (P)	1 (VPS13B,M,-)	0	3 (P,f)(P,***)(M,-)

3.3 De novo and inherited variants

It was possible to perform family segregation analysis in 74 out of 86 patients, for 2 patients only the mother was available for segregation analysis. We found 4 de novo (Table 6) and 288 inherited variants.

Table 6- De novo Variants

Patient	*Gene*	*Gene SFARI score*	*Variant*	*Inheritance*	*Predictions*	*Reference*	*Allele frequency (GnomAD)*
A14/55	*ANKRD11*	1	c.890C>T, p.Thr297Met	*De novo*	S damaging, P probably damaging	/	0,0012 (1/800)
	NF1	1	c.1985A>G, p.Lys662Arg	*De novo*	S tolerated; P benign	/	0,00002 (1/50000)
L14/95	*SLIT3*	no rating	c.1886G>A, p.Ser629Asn	*De novo*	S tolerated; P benign	Cukier *et al.* 2014	0,007 (1/140)
LDM167	*PTEN*	1	c.1131_1132dupTA, p.Arg378IlefsTer39	*De novo*	S /, P /	/	not present

S, SIFT; P, Poliphen

As for de novo variants, two of them were found in the same patient (A14/55), for whom no other candidate variants were identified. The first variant is located in the *ANKRD11* gene, it is frequent and predicted probably damaging by Polyphen. The second variant is located in *NF1* gene, it is rare and predicted tolerated by SIFT and benign by Polyphen. In patient L14/95 we found a frequent, tolerated, and benign variant in *SLIT3* gene. This variant has already been associated with ASD as described by Cukier et al. (2014). In patient LDM167 we detected a small duplication of two nucleotides (c.1131_1132dupTA) in *PTEN* gene that causes the formation of a premature stop codon. This variant is not present in GnomAD database and is predicted to be disease causing by Mutation taster.

As for inherited variants, we observed that all variants identified in our cohort came equally from the paternal and maternal genetic makeup. This equal contribution is observed also if we consider only extremely rare variants (***,**) (72 paternal and 72 maternal)(Figure 5).

Table 7 - Variants already described in other studies

Gene	*Gene SFARI score*	*Variant*	*Inheritance*	*Predictions*	*Reference*	*Allele frequency (GnomAD)*	*Patient*
ASTN2	2	c.769T>A, p.Ser257Thr	father	Damaging (S), Possibly damaging (P)	Lionel et al., (2014)	2,05x10-3 (1/480) (f)	D17/50
ATRX	1	c.1825C>G, p.Pro609Ala	Father	unknown (S), benign (P)	Brett M, et al. (2014)	1x10-3 (1/1000) (*)	P.J.A.-84366
CACNA1H	2	c.6322G>A, p.Ala2108Thr	Father	Tolerated (S), Benign (P)	Chourasia N, et al. (2019)	1,42x10-3 (1/700) (f)	G17/11
CADPS2	3	c.1889T>C, p.Met630Thr	mother	Damaging (S), Benign (P)	Bonora et al. (2014)	6,26x10-4 (1/1600) (*)	LDM1089
CEP290	3S	c.1079G>A, p.Arg360Gln	Father	unknown (S), Possibly damaging (P)	Deciphering Developmental Disorders Study (2014)	5,15x10-3 (1/190) (f)	D.G.M.-50685
CREBBP	1	c.2941G>A, p.Ala981Thr	Unknown	unknown (S), unknown (P)	Coupry et al. (2002)	0,003 (1/300) (f)	D10/14
FAT1	3	c.12653 A>G, p.Asp4218Gly	Mother, father, father	Tolerated (S), Probably damaging (P)	Cukier et al. (2014)	1,02x10-2 (1/98) (f)	r.g.74126, LDM733, 70102
GABRB3	1	c.31C>T, p.Pro11Ser	father	Tolerated (S), Benign (P)	Delahanty RJ, et al. (2009); Alvarez-Mora MI, et al. (2016)	2,95x10-3 (1/330) (f)	75323
MBD5	3	c.236G>A, p.Gly79Glu	Unknown	Deleterious (S), Probably damaging (P)	Tolkowski et al. (2011)	0,0005 (1/2000) (*)	C.L.
NRXN1	1	c.2242C>A, p.Leu748Ile	Mother, Father	Tolerated (S), Possibly damaging (P)	Kim HG , et al. (2008) Onay H , et al. (2017) Reale et al. (2021)	0,004 (1/250) (f)	O12/11, D17/19
PTEN	1	c.235G>A, p.Ala79Thr	Father, Father	Tolerated (S), Benign (P)	Aspromonte MC , et al. (2019)	1,03x10-4 (1/9700) (**)	C17/76, LDM568
RELN	1	c.5156C>T, p.Ser1719Leu	Father	Deleterious (S), Probably damaging (P)	Bonora et al. 2003	0,006 (1/150) (f)	A14/06
RELN	1	c.7438G>A, p.Gly2480Ser	Mother	Tolerated (S), Possibly damaging (P)	Bonora et al. 2003	$2,62x10^{-3}$ (1/380) (f)	M.S.-64656
RELN	1	c.3477C>A, p.Asn1159Lys	father	Tolerated (S), Possibly damaging (P)	Bonora et al. 2003	1,56x10-3 (1/178) (f)	80147
SCN1A	1	c.1811G>A, p.Arg604His	Mother	Tolerated (S), Possibly damaging (P)	Alvarez-Mora MI, et al. (2016)	1,38x10-3 (1/700) (f)	73206
SCN1A	1	c.133G>A, p.Asp45Asn	Father	Tolerated (S), Benign (P)	Carvill GL, et al. (2013)	7,95x10-6 (1/125000) (***)	52984
SLC6A4	3	c.167G>C, p.Gly56Ala	Father, Father	Damaging (S), Benign (P)	Sutcliffe et al. 2005, Adamsen et al. 2010, Reale et al. (2021)	1,18x10-2 (1/84) (f)	D15/81, 75323
SLIT3	no rating	c.1886G>A, p.Ser629Asn	*De Novo*	Tolerated (S), Benign (P)	Cukier et al. 2014	0,007 (1/140) (f)	L14/95
TSC1	1	c.1960 C>G, p.Gln654Glu	Mother, Mother	Tolerated (S), Benign (P)	Kelleher et al. (2012)	7,9x10-4 (1/1200) (*)	H.S.M., P.J.A.-84366
VPS13B	1	c.1832G>A, p.Arg611Lys	Mother	Tolerated (S), Benign (P)	Ionita-Laza I , et al. (2014)	1,27x10-3 (1/780) (*)	P.A.-79782
VPS13B	1	c.1559A>G, p.His520Arg	mother	Deleterious (S), Probably damaging (P)	Ionita-Laza I, et al. (2014)	5,81x10-4 (1/1700) (*)	52724

S,SIFT; P, Polyphen2, (***), X<10000; (**) ,1/10000<X<1/5000;(*), 1/5000<X<1/1000; (f), 1/500<x<1/100

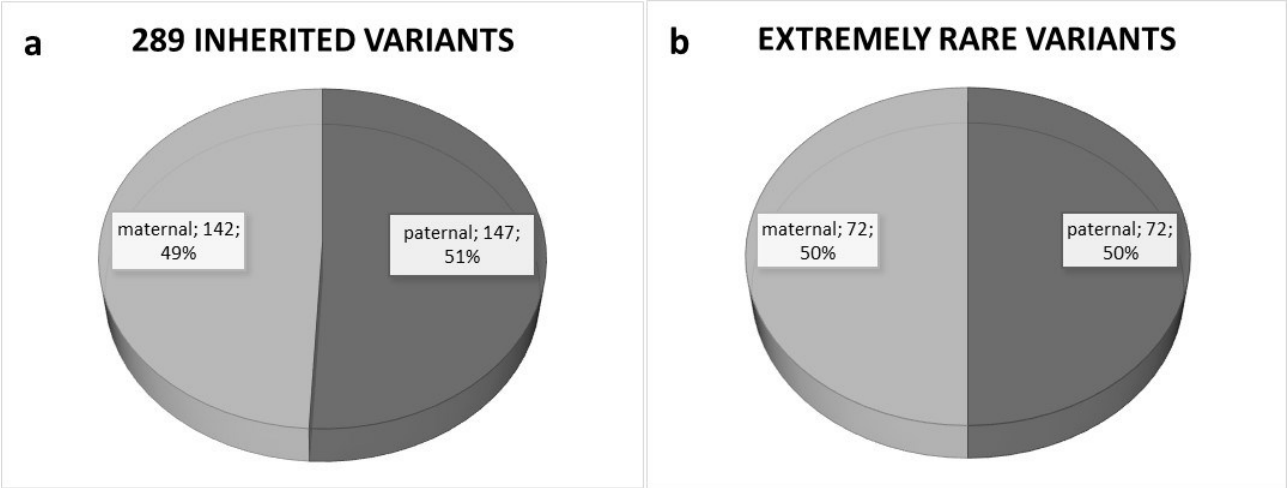


Figure 5- Graphic representation of segregation analysis data of all inherited variants (a). Graphic representation of segregation analysis data of inherited extremely rare variants (b)

3.3 Variants already associated with Autism

We found 21 variants that were already described in literature and associated with Autism **(**Table 7**)**.

Most of these variants are relatively frequent, in fact we found 6 variants with frequency between 0.02% and 0.1% (*) and 13 variants with frequency between 0.2% and 1.0% (f). We found two extremely rare variants.

We found that 4 of these variants are recurrent in 2 patients of our cohort, and 1 is present in 3 patients. Of these recurrent variants already described, one is extremely rare.

In two cases it was not possible to segregate the variants, while one is de novo and the others are inherited. In two patients we found two already described variants (75323, P.J.A.-84366).

The variant c.2242C>A (p.Leu748Ile) in *NRXN1* gene was already found in 2 patients with severe autism and paternal transmission (Onay et al, 2017) (Talkowski et al, 2011) In one patient (O12/11) we found this variant, inherited from the mother, in the other (D17/19) inherited from the father. Both patients have 4 other variants.

The known Gly56Ala mutation (c.167G>C) in the serotonin transporter SERT (or 5-HTT), encoded by the *SLC6A4* gene, causes increased serotonin reuptake and has been associated with autism and rigid-compulsive behavior (Sutcliffe et al, 2005) (Kelleher et al, 2012). We found this variant in two patients (D15/81, 75323), both paternally inherited.

FAT1 gene was identified in one ASD family in a whole-exome sequencing study, as described by Cukier et al (2014). In our cohort, this variant was found in 3 patients (r.g.74126, LDM733, 70102), in one case inherited from the mother, in the other two cases paternally inherited.

The variant c.1886G>A (p.Ser629Asn) in *SLIT3* gene was identified in two ASD families in a whole exome sequencing study, as described by Cukier et al. (2014). In our study, the p.Ser629Asn was found in one patient (L14/95) as a de novo variant, together with two not extremely rare variants (in *MBD5*, AF 0,2%, and *VPS13B*, AF 0,3%) and two more rare variants (in *ANK2*, AF 0,07%, and *KDM6B*, AF 0,02), all maternally inherited.

The variant c.1960C>G (p.Gln654Glu) in *TSC1* gene was firstly identified in a study by Kelleher et al. (2012), in our cohort we found this variant in two patients (H.S.M. and P.J.A.-84366), carried in both by the maternal allele. In both patients other variants were found: in H.S.M three variants, all inherited, 2 paternally and one maternally inherited; in P.J.A.-84366 other 5 variants, 2 paternally inherited and 3 maternally inherited.

The variant c.235G>A, (p.Ala79Thr) in *PTEN* gene has been described by Aspromonte et al (2019) in a cohort of patients with neurodevelopmental disorders, mainly ID and ASD; the variant, maternally inherited, has been interpreted as of unknown significance, and partially related to phenotype. In our sample the interesting thing is that it has been found in a multiplex family, in two siblings (C17/76, LDM568), female and male, paternally inherited. In each patient we found

other 4 variants each, all inherited, and only one other is shared by the two probands (see Table 8).

3.4 Recurrent variants

We identified 19 out of 336 variants shared by two patients, 6 out of 336 shared by 3 patients and 2 shared by 4 patients. Two patients share two variants, one in *ZEB2* and one in *RAI1*.

Most of them are relatively common, but we found 4 very rare variants, one in *NRXN3*, c.3156G>T (p.Gln1052His) and one in NSD1 gene c.7367T>C (p.Met2456Thr).

The others in *CACNA1H* gene, c.4685G>A (p.Arg1562Gln) and in *PTEN* gene, c.235G>A; p.Ala79Thr, were found in two siblings, both inherited, the first from the mother, and the second from the father, as already described in the previous paragraph.

We also found a recurrent variant in *ANKRD11* gene, c.890C>T; p.Thr297Met, that is the unique recurrent variant that segregation analysis found *de novo* in one patient, found with another de novo variant in *NF1* gene (A14/55). In the other patient (P.A.-79782) it was inherited from the mother, and in this patient we found also other 4 variants, all inherited. Notably, these two patients come from different world regions (the first North African, the second Italian).

Table 8- Variants shared by more than one patient

Gene	SFARI Score	Variant	Chr position (GRCh37)	Predictions (S-P)	GnomAD	Patient	Transmission
MBD5	1	c.2030G>A; p.Ser677Asn	2:149227542	S tolerated, P benign	2,23x10-3 (1/450)	L14/95	Maternal
						52984	Paternal
						A17/97	Paternal
						D17/27	Paternal
ANKRD11	1	c.5338G>A; p.Ala1780Thr	16:89347612	S tolerated, P benign	4,86x10-3 (1/200)	O12/11	Paternal
						73206	Paternal
						75544	Maternal
						E17/49	Maternal
SCN2A	1	c.2723A>G; p.Lys908Arg	2:166201225	S tolerated, P possibly damaging	4,21x10-3 (1/230)	C.L.	unknown
						IB17A	Maternal
						73400	Paternal
CEP290	3S	c.6401T>C; p.Ile2134Thr	12:88454728	S damaging, P probably damaging	6,99x10-3 (1/140)	G14/62	Maternal
						N13/03	Maternal
						70555	Paternal
RAI1	1	c.1142C>T; p.Ala381Val	17:17697404	S deleterious, P benign	0,004 (1/250)	r.g.74126	Paternal
						a.d.81815	Paternal
						C.P.P	Maternal
ZEB2	/	c.2230A>G; p.Ile744Val	2:145156524	S tolerated, P benign	0,002 (1/500)	r.g.74126	Paternal
						a.d.81815	Maternal
						C.L.	Maternal
DLGAP2	3	c.1751C>T; p.Thr584Met	8:1616675	S tolerated, P benign	5,8x10-3 (1/172)	N13/03	Paternal
						C17/76	Maternal
						79149	Maternal
FAT1	3	c.12653 A>G	4:187518041	S tolerated, P probably damaging	1,02x10-2 (1/98)	r.g.74126	Maternal
						LDM733	Paternal
						70102	Paternal
FAT1	3	c.4841C>T; p.Pro1614Leu	4:187542899	S damaging, P possibly damaging	0,003 (1/300)	H11/97	unknown
						M14/99	unknown
VPS13B	1	c.2485G>A; p.Ala829Thr	8:100205255	S tolerated, P benign	0,0085 (1/115)	D15/81	Paternal
						LDM167	Maternal
VPS13B	1	c.1639A>G; p.Thr547Ala	8:100148968	S tolerated, P benign	$2,26x10^{-4}$ (1/4400)	C.M.L.-63035	Paternal
						D17/19	Paternal
VPS13B	1	c.7753G>A; p.Glu2585Lys	8:100791158	S tolerated, P benign	3,15x10-3 (1/300)	LDM632	Paternal
						73664	Paternal
SLC6A4	3	c.167G>C; p.Gly56Ala	17:28548810	S tolerated, P benign	1,18x10-2 (1/84)	D15/81	Paternal
						75323	Paternal

Gene	SFARI Score	Variant	Chr position (GRCh37)	Predictions (S-P)	GnomAD	Patient	Transmission
CADPS2	3	c.1889T>C; p.Met630Thr	7:122114544	S damaging, P benign	6,26x10-4 (1/1600)	N13/03	Maternal
						LDM1089	Maternal
TSC1	1	c.1960 C>G p.Gln654Glu	9:135781005	S tolerated, P benign	7,9x10-4 (1/1200)	H.S.M.	Maternal
						P.J.A.-84366	Maternal
NRXN1	1	c.2242C>A; p.Leu748Ile	2:50765412	S tolerated, P possibly damaging	3,98x10-3 (1/250)	O12/11	Maternal
						D17/19	Paternal
FAT1	3	c.2563G>A; p.Gly855Arg	4:187628419	S damaging, P probably damaging	2,11x10-3 (1/470)	s.s.73499	Paternal
						D17/27	Maternal
PCDH9	3	c.2714G>C; p.Ser905Thr	13:67799859	S tolerated, P possibly damaging	not present	D.G.M.-50685	Paternal
						85256	Maternal
NRXN3	1	c.3156G>T; p.Gln1052His	14:80328277	S damaging, P benign	7,85x10-5 (1/12700)	H16/06	Paternal
						D17/50	Maternal
PTEN	1	c.235G>A; p.Ala79Thr	10:89690828	S tolerated, P benign	1,03x10-4 (1/9700)	C17/76	Paternal (*)
						LDM568	Paternal (*)
CACNA1H	2	c.4685G>A; p.Arg1562Gln	16:1262064	S deleterious, P probably damaging	1,21x10-5 (1/82600)	C17/76	Maternal (*)
						LDM568	Maternal (*)
CACNA1H	2	c.5113G>A; p.Ala1705Thr	16:1265315	S tolerated, P probably damaging	5,51x10-3 (1/180)	D15/81	Paternal
						70555	Maternal
ANKRD11	1	c.5509C>T; p.Pro1837Ser	16:89347441	S tolerated, P benign	4,11x10-3 (1/240)	E17/49	Paternal
						D17/35	Paternal
MED12	no rating	c.5711C>T; p.Ala1904Val	X:70357196	S /, P benign	1,11x10-3 (1/900)	LDM568	Paternal
						LDM565	Maternal
ANKRD11	1	c.890C>T; p.Thr297Met	16:89352449	S damaging, P probably damaging	1,24x10-3 (1/800)	A14/55	De novo
						P.A.-79782	Maternal
NSD1	1	c.7367T>C; p.Met2456Thr	5:176721736	S deleterious -low confidence, P benign	0,00006 (1/16000)	F14/70	Maternal
						B12/69	Paternal
CSMD1	3	c.8935G>A; p.Gly2979Ser	8:2824257	S damaging, P probably damaging	5,45x10-3 (1/183)	r.g.74126	Maternal
						70555	Maternal

S,SIFT; P, polyphen

(*) same family

3.5 More variants in the same gene

We found 2 variants in the same gene in 12 patients (Table 10). The *FAT1* gene was found mutated twice in 3 different patients (F12/67, D17/27 and LDM733), as *VPS13B* (A15/18, 70555 and D10/14).

In one patient (M.S.-64656) we found three variants in *CEP290* gene all paternally inherited, and in the same patient we found 2 variants in *ANK2*, both maternally inherited.

In patient G14/62 we found 2 variants in *ZNF804A*, not inherited by the mother. Also, in patient H16/64, two variants located on the same allele of *CHD8* were found both paternally transmitted. In patient E11/61 we found two mutations in *GNA14* but was not possible to study segregation in the family. We also found a homozygous mutation in *MET* gene in patient F14/70, transmitted by both parents.

3.6 Most frequently mutated genes

We identified 6 genes that are more frequently mutated respect to the others, with more than 10 variants found in each of them.

Respect of our previous work (Reale et al, 2021), we confirm the presence of high rate of variants in 5 (*FAT1, VPS13B, ANK2, ANKRD11* and *CSMD1*), with more than 10 variants present in the whole cohort. We found a new gene that had more than 10 variants, *CACNA1H.*

Table 9- Most frequently mutated genes in our cohort

Gene	*SFARI score*	*number of variants*	*patients*	*same variants*	*ASD frequency in our cohort*	*GnomAD frequency*	*Statistical significance exact p-value (1-tailed)*
VPS13B	1	25	22	3	0,256	0,000062	exact *p*<.0001
FAT1	3	19	16	3	0,186	0,000061	exact p<.0001
ANKRD11	1	16	15	3 (one in 4 patients)	0,174	0,00004	exact *p*<.0001
CSMD1	3	11	11	1	0,128	0,000038	exact *p*<.0001
CACNA1H	2	11	11	2	0,128	0,000024	exact p<.0001
ANK2	1	10	9	none	0,11	0,000046	exact p<.0001

In detail, we detected 25 variants in *VPS13B* gene, three shared by two patients (Table 8), moreover, in 3 patients (D10/14, A15/18 and 70555) we found 2 variants of this gene (Table 10).

We found 19 variants in *FAT1* gene, and in detail we found three recurrent variants (two variants detected twice, and one detected in three patients, see Table 8) and in three patients *(*F12/67, D17/27, LDM733*)* we found two variants in *FAT1* gene in the same patient, in two cases (F12/67, LDM733) paternally inherited, in one case inherited from both parents (D17/27).

We found 16 variants in *ANKRD11* gene, of which three repeated in our cohort (one fund in 4 patients, 2 found twice, and one is de novo); in one patient (E17/49) we found two *ANKRD11* gene variants.

We moreover found 11 variants in *CSMD1* and *CACNA1H* gene and 10 variants in *ANK2* gene.

We used a binomial test to compare the mutational frequencies of these 6 genes in our cohort with the frequencies in GnomAD population (as shown in Table 9). For all these 6 genes we noted significantly increased frequencies of rare variants in our study population. In detail, the results indicate that the frequency of mutation in all the genes we take into consideration is higher than expected (1-tailed exact $p<0,0001$).

Table 10-Variants that are in the same gene and in the same patient

Gene	SFARI Score	Variant	Chr position (GRCh37)	Predictions (S-P)	GnomAD	Patient	Transmission
VPS13B	1	c.11270G>A; p.Arg3757Gln	8:100874154	S tolerated , P possibly damaging	0,0017 (1/600)	D10/14	unknown
		c.11825_11827dupATG; p.Asp3942dup	8:100887648	S /, P /	0,00045 (1/2500)	D10/14	unknown
VPS13B	1	c.611A>G; p.Asn204Ser	8:100123356	S tolerated, P possibly damaging	0,00009 (1/11000)	A15/18	unknown
		c.6304C>T; p.Arg2102Cys	8:100711935	S tolerated, P benign	0,000008 (1/125000)	A15/18	unknown
VPS13B	1	c.5282A>G; p.Thr1271Ser	8:100493971	S tolerated; P probably damaging	7,22x10-4 (1/1385)	70555	Maternal
		c.5282A>G; p.Ser2596Phe	8:100791192	S damaging, P possibly damaging	7,27x10-4 (1/1375)	70555	Maternal
FAT1	3	c.9457G>A; p.Asp3153Asn	4:187534269	..., P probably damaging	not present	F12/67	Paternal
		c.7957G>A; p.Gly2653Ser	4:187539783	S deleterious , P probably damaging	0,0006 (1/1500)	F12/67	Paternal
FAT1	3	c.2563G>A; p.Gly855Arg	4:187628419	S damaging, P probably damaging	2,11x10-3 (1/470)	D17/27	Maternal
		c.56G>C; p.Gly19Ala	4:187630926	S tolerated, P benign	3,68x10-4 (1/2700)	D17/27	Paternal
FAT1	3	c.12653A>G; p.Asp4218Gly	4:187518041	S tolerated; P probably damaging	1,02x10-2 (1/98)	LDM733	Paternal
		c.4358G>A; p.Arg1453His	4:187549883	S damaging; P probably damaging	1,5x10-3 (1/660)	LDM733	Paternal
ANKRD11	1	c.5282A>G; p.Pro1837Ser	16:89347441	S tolerated; P benign	4,11x10-3 (1/240)	E17/49	Paternal
		c.5282A>G; p.Ala1780Thr	16:89347612	S tolerated; P benign	4,86x10-3 (1/200)	E17/49	Maternal
CHD8	1	c.5282A>G; p.Lys1761Arg	14:21863179	S tolerated; P benign	not present	H16/64	Paternal
		c.5282A>G; p.Pro615Ser	14:21883940	S tolerated; P benign	1,77x10-3 (1/560)	H16/64	Paternal
CEP290		c.5998A>G; p.Ile2000Val	12:88465084	S/, P benign	$1,72x10^{-4}$ (1/1300)	M.S.-64656	Paternal
		c.1092T>G; p.Ile364Met	12:88519120	S/, P probably damaging	$8,44x10^{-4}$ (1/1100)	M.S.-64656	Paternal
		c.963T>A; p.Asp321Glu	12:88520195	S/, P probably damaging	$9,13x10^{-5}$ (1/10100)	M.S.-64656	Paternal
ANK2	1	c.6854T>C; p.Ile2285Thr	4:114276628	S/, P benign	$1,25x10^{-4}$ (1/8000)	M.S.-64656	Maternal
		c.11716C>T; p.Arg3906Trp	4:114294462	S/, P probably damaging	$1,07x10^{-3}$ (1/900)	M.S.-64656	Maternal
ZNF804A	2	c.735_737delATT; p.Phe246del	2:185800857	S /, P /	0,0005 (1/2000)	G14/62	Maternal
		c.1490T>C; p.Leu497Pro	2:185801613	S deleterious, P benign	0,0004 (1/2500)	G14/62	Maternal
GNA14	no rating	c.215C>T; p.Thr72Met	9:80144079	S tolerated , P possibly damaging	0,0013 (1/750)	E11/61	unknown
		c.97C>T; p.Arg33Cys	9:80262613	S deleterious, P benign	0,005 (1/200)	E11/61	unknown

According to correlation with behavioral phenotype, we did not find any statistical significance for the analysis performed. In particular, the number of variants was not influenced by familiar history for neuropsychiatric disorders and was not related to cognitive and behavioral features.

3.7 Clustering

We analyzed for each patient with more than 5 variants (30 patients) the functional protein association networks using STRING (string-db.org)

We found statistical significance in protein networks in 9 out of 30 patients (see Table 11 and figures 6-14).

In one case (C15/87) we found significance for the network but no functional enrichment.

For most of these networks, however, enrichment significance was due to PubMed literature co-reports; in few there seemed to be more a functional involvement of greater interest.

This enrichment is linked to keyword as "autism" and related terms, "intellectual disability" and related and to "genetic disease" (Table 12, Table 13).

In one patient (A14/06) we found a high significance, due to the presence of variants in neurexin and neuroligins genes. In this patient we found a strong literature related to the functional network, and the presence of significance in biological processes related to synaptic function and molecular processes related to neuroligin family protein binding (Table 14). The interesting thing, moreover, is that these variants are maternally (*NRXN1, NRXN3, VPS13B*) and paternally (*ZNF804A, RELN, RAI1*) inherited, so that this network is present only in the proband.

In another patient, 14/70, we found an interesting finding that seem to have a strong correlation in a network related to *ASH1L* gene (Table 14).

Table 11 - Network Statistics

Id Patient	Number Of Nodes	Number Of Edges	Average Node Degree	Average Local Clustering Coefficient	Ppi Enrichment P-Value	Figure
C17/76	5	2	0.8	0.4	0.0483	Figure 11
73206	8	3	0.75	0.25	0.0074	Figure 8
D17/27	7	2	0.571	0.571	0.0118	Figure 10
F14/70	5	4	1.6	0.667	0.0000241	Figure 6
D17/19	5	4	1.6	0.667	0.0000316	Figure 13
O12/11	5	3	1.2	0.6	0.000138	Figure 7
A14/06	6	4	1.33	0.556	0.0000206	Figure 9
M.S.-64656	9	3	0.667	0.667	0.00403	Figure 14
C15/87	5	2	0.8	0.4	0.00358	Figure 12

Table 12- STRING pathway enrichments enrichment in Keyword

ID patient	#term ID	term description	observed gene count	background gene count	strength	false discovery rate	matching proteins in your network (labels)
F14/70	KW-0160	Chromosomal rearrangement	3	307	1.58	0.0355	*MED13L, MET,NSD1*
F14/70	KW-0158	Chromosome	3	421	1.45	0.0452	*POGZ, ASH1L,NSD1*
F14/70	KW-0225	Disease mutation	5	3145	0.79	0.0452	*POGZ, MED13L,MET,ASH1L,NSD1*
F14/70	KW-0991	Mental retardation	3	468	1.4	0.0452	*POGZ, MED13L,ASH1L*
73206	KW-1269	Autism	2	25	2.29	0.0472	*SCN1A, SHANK2*
M.S.-64656	KW-0991	Mental retardation	5	468	1.37	0.00087	*POGZ, NIPBL,ANKRD11,SATB2,CEP290*
M.S.-64656	KW-0225	Disease mutation	7	3145	0.68	0.0343	*POGZ, NIPBL,ANKRD11,ANK2,RELN,SATB2,CEP290*
A14/06	KW-0245	EGF-like domain	3	229	1.63	0.0296	*NRXN1, RELN,NRXN3*
O12/11	KW-0991	Mental retardation	5	468	1.62	7.46e-06	*ANKRD11, CNTNAP2,CDKL5,NRXN1,SOX5*

Table 13 - STRING pathway enrichments enrichment in Disease-gene association

ID patient	#Term ID	term description	observed gene count	background gene count	strength	false discovery rate	matching proteins in your network (labels)
C17/76	DOID:0060041	Autism spectrum disorder	3	34	2.54	0.00027	*CACNA1H, PTEN,CNTN4*
C17/76	DOID:0060037	Developmental disorder of mental health	4	514	1.48	0.0035	*CACNA1H, PTEN,CNTN4,EHMT1*
M.S.-64656	DOID:0060037	Developmental disorder of mental health	7	514	1.47	1.36e-06	*POGZ, NIPBL,ANKRD11,ANK2,RELN,SATB2,CEP290*
M.S.-64656	DOID:0060041	Autism spectrum disorder	3	34	2.28	0.00073	*POGZ, ANK2,RELN*
M.S.-64656	DOID:1059	Intellectual disability	5	412	1.42	0.00073	*POGZ, NIPBL,ANKRD11,SATB2,CEP290*
M.S.-64656	DOID:630	Genetic disease	8	2962	0.77	0.0014	*POGZ, NIPBL,ANKRD11,RAI1,ANK2,RELN,SATB2,CEP290*
O12/11	DOID:1059	Intellectual disability	5	412	1.68	1.87e-05	*ANKRD11, CNTNAP2,CDKL5,NRXN1,SOX5*
O12/11	DOID:0060040	Pervasive developmental disorder	3	37	2.5	6.89e-05	*CNTNAP2, CDKL5,NRXN1*
O12/11	DOID:12849	Autistic disorder	2	8	2.99	0.0017	*CNTNAP2, NRXN1*

Table 14 - STRING pathway enrichments enrichment in Molecular Process (Gene Ontology)

ID patient	#Term ID	term description	observed gene count	background gene count	strength	false discovery rate	matching proteins in your network (labels)
F14/70	GO:0046975	Histone methyltransferase activity (h3-k36 specific)	2	11	2.85	0.0213	*ASH1L, NSD1*
A14/06	GO:0097109	Neuroligin family protein binding	2	5	3.12	0.0086	*NRXN1, NRXN3*

Table 15 - STRING pathway enrichments enrichment in Biological Process (Gene Ontology)

ID patient	#Term ID	term description	observed gene count	background gene count	strength	false discovery rate	matching proteins in your network (labels)
O12/11	GO:0042297	Vocal learning	2	6	3.12	0.0256	*CNTNAP2, NRXN1*
O12/11	GO:0071625	Vocalization behavior	2	17	2.66	0.0256	*CNTNAP2, NRXN1*
C17/76	GO:0007270	Neuron-neuron synaptic transmission	2	8	2.99	0.0411	*PTEN, DLGAP2*
A14/06	GO:0090129	Positive regulation of synapse maturation	2	9	2.86	0.0206	*NRXN1, RELN*
A14/06	GO:0097114	NMDA glutamate receptor clustering	2	4	3.21	0.0206	*NRXN1, RELN*
A14/06	GO:0097119	Postsynaptic density protein 95 clustering	2	6	3.04	0.0206	*NRXN1, RELN*
A14/06	GO:0099175	Regulation of post synapse organization	3	107	1.96	0.0206	*ZNF804A, NRXN1,RELN*
A14/06	GO:0007612	Learning	3	145	1.83	0.0243	*NRXN1, RELN,NRXN3*
A14/06	GO:0007158	Neuron cell-cell adhesion	2	16	2.61	0.0262	*NRXN1, NRXN3*
A14/06	GO:0071625	Vocalization behavior	2	17	2.58	0.0262	*NRXN1, NRXN3*
A14/06	GO:0051968	Positive regulation of synaptic transmission, glutamatergic	2	26	2.4	0.0400	*NRXN1, RELN*
A14/06	GO:2000311	Regulation of ampa receptor activity	2	25	2.42	0.0400	*NRXN1, RELN*
A14/06	GO:2000463	Positive regulation of excitatory postsynaptic potential	2	28	2.37	0.0400	*NRXN1, RELN*

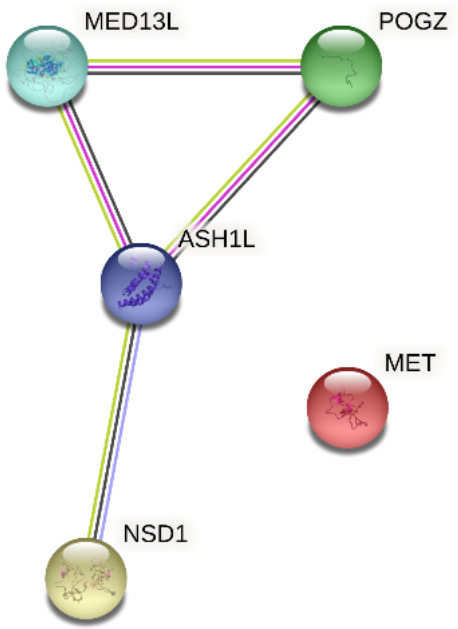


Figure 6 - functional protein association network (ID F14/70)

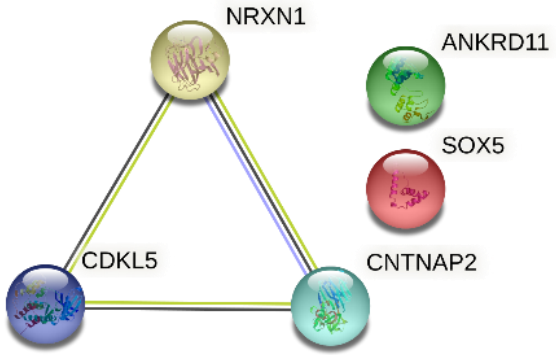


Figure 7 - functional protein association network (ID O12/11)

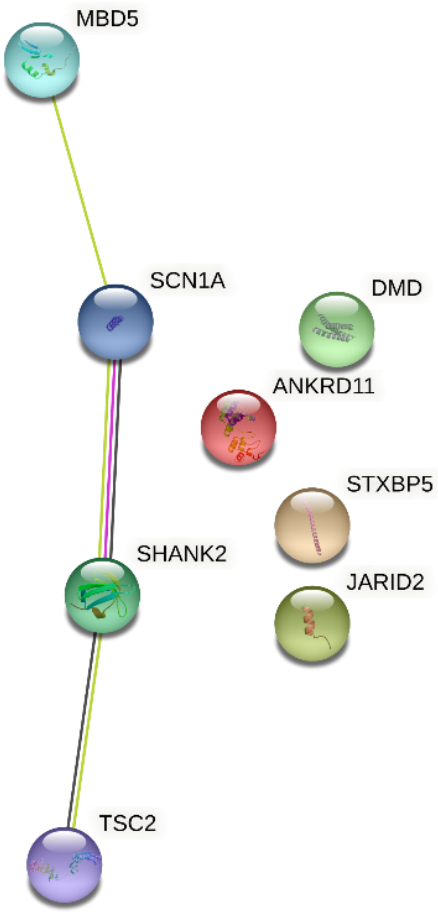


Figure 8 - functional protein association network (ID 73206)

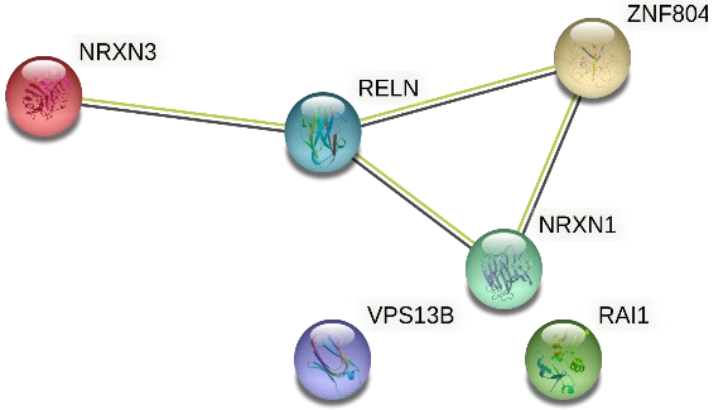


Figure 9 - functional protein association network (ID A14/06)

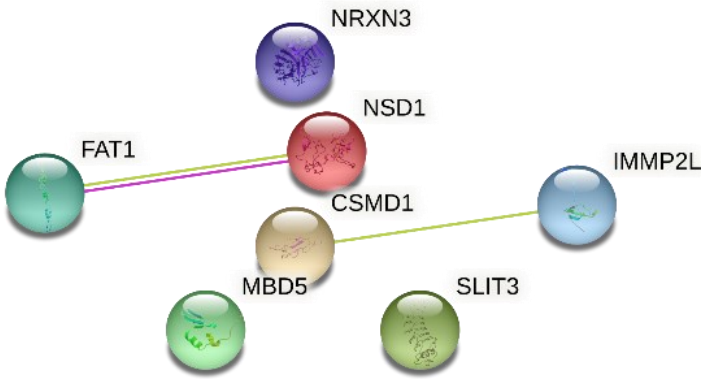


Figure 10 - functional protein association network (ID D17/27)

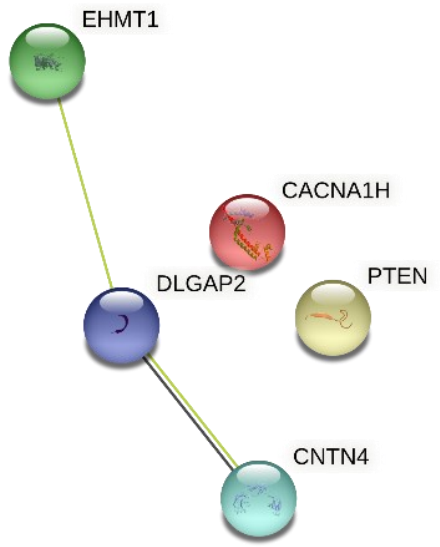


Figure 11 - functional protein association network (ID C17/76)

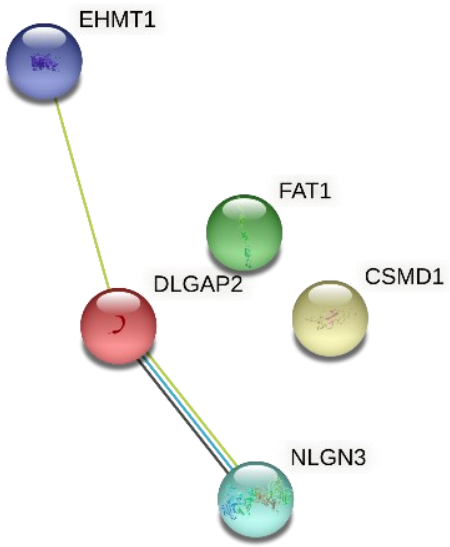


Figure 12- functional protein association network (ID C15/87)

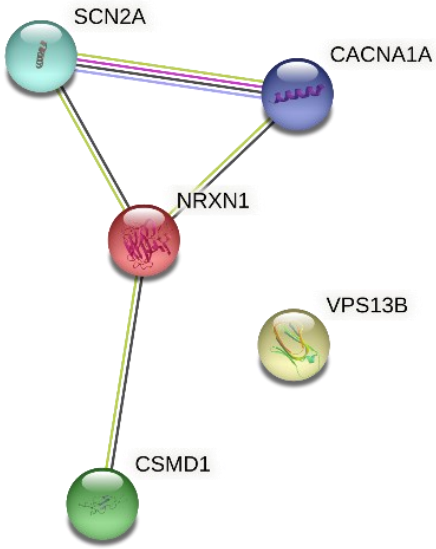


Figure 13 - functional protein association network (ID D17/19)

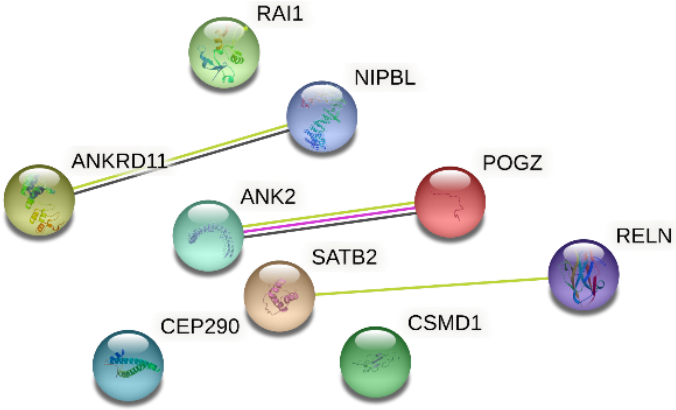


Figure 14 - functional protein association network (ID M.S.-64656)

Legend

- From curated databases
- Experimentally determined
- Text mining
- Gene co-occurrence
- Co-expression
- Protein homology
- Gene fusions
- Gene neighborhood

Edges represent protein-protein associations

associations are meant to be specific and meaningful, i.e., proteins jointly contribute to a shared function; this does not necessarily mean they are physically binding to each other.

4. Discussion

We analyzed the coding regions of 120 ASD candidate genes in 86 autism patients to identify novel mutations, risk genes and new genotype-phenotype associations.

The diagnostic yield of our targeted gene panel is lower than estimated in literature (12-14%)(Alvarez-Mora et al, 2015, Kalsner et al, 2018). We found in fact only one variant considered pathogenic (*de novo* variant in *PTEN* in LDM167 subject). It is therefore quite difficult to compare different studies for the genes selected in the panels and for the populations studied.

Before any dissertation about the results obtained, we must underline that even if considering only rare variants, most variants found in our sample are missense and inherited mutations, that make our results weak on direct pathogenetic impact, but worth of reflection about their possible meaning and perspective on ASD genetic basis.

We decided to focus on the detection of rare variants of any type. This is because, when genetic variants are extremely rare or not reported in current databases, such as gnomAD (Lek et al, 2016), this might suggest a possible role in ASD and neurodevelopmental/psychiatric disorders, since low reproductive rate is highly associated with these disorders. (Lovato et al, 2019).

The first aspect of interest is the presence of a high number of variants in the subjects tested. In fact, among the 86 patients' samples screened, only 2 subjects had no variants detected, and four resulted carriers of a unique candidate variant, while 80 patients were found to be carrier of more than one variant. Moreover, in most patients (60 subjects) we found 4 or more variants. This result is very interesting,

especially because the panel used allows the analysis of only 120 genes, despite the approximately one thousand genes currently associated with ASD.

The other interesting aspect is the low rate of de novo variants. Of the 295 variants whose segregation has been studied, we found only 4 *de novo* variants in 4 different genes (*ANKRD11*, *NF1*, *SLIT3* and *PTEN)* in 3 patients. This datum (1,3%) is remarkably lower than the ratio published in the literature (according to De Rubeis *et al.* (2014), *de novo* loss-of-function mutations are in over 5% of ASD patients).
All four genes are reported in literature as autism risk genes or genes causing syndromes in which subgroups of patients may develop autism (Iossifov et al, 2014, Zhang et al, 2009, Iossifov et al, 2015).The interesting thing is that two of them (*ANKRD11* and *PTEN*) involve genes that have been recently described as implicated in risk for autism spectrum disorder (ASD genes) in a large study that involved thousands of subjects (Satterstrom et al, 2020); moreover, *de novo* variant in *ANKRD11* gene was found in a patient carrier of another *de novo* variant, in *NF1* gene (scoring 1 in SFARI database).
As for *PTEN* variant, it is to note that the only clinical finding in the patient was the presence of mild macrocephaly (cranial circumference 97°percentile) with height at 75° percentile.
As for the other de novo variant, in *SLIT3*, it was found in a patient carrier of other 4 maternal inherited variants in genes of interest (*MBD5, ANK2, VPS13B, KDM6B*).
Possible explanations of the low rate of *de novo* variants could probably be due to the small number of genes analyzed in our cohort, or it could derive from recruitment criteria in our cohort, namely from the exclusion of patients with syndromic characteristics, more frequently found to be caused by *de novo* CNV or variants in genes already associated with other syndromes. In fact, in line with our previous work (Annunziata et al, 2021) and as reported in literature (Toriello, 2012;

Jacquemont et al., 2006; Lovrečić et al., 2018), we found a lower rate of *de novo* events in subjects with essential autism spectrum disorder.

As we know, the increased in *de novo* events in subjects with ASD is widely described in literature (Chaste et al, 2017) and several studies highlighted the importance of *de novo* mutations in a situation of family mutational burden due to the presence of common and rare variants inherited from parents. (Pinto et al. 2010; Ronemus M., Iossifov I. et al. 2014).

Another possible explication derives from new insight into autism genetics, that deals with the possible role of private inherited variants. Recently, Wilfert and colleagues (2021) studied a wide cohort of over 3000 families, with particular focus on transmission disequilibrium of private, likely gene-disruptive variants, and found that they appear to act on a distinct set of genes, not yet associated with autism, as most resides outside of known *de novo* mutation-enriched genes. Moreover, they estimate that these variants are significantly younger than other variants of similar type and frequency in siblings, and that they more strongly affect multiplex family probands, supporting a multi-hit model for ASD. These private Likely Gene Disrupting variants, preferentially transmitted to probands, converge on functional networks already described in ASD. The interesting thing is the assumption that we could consider inherited variants as sort of "recent *de novo*" variants, that confer lesser risk than *de novo* variants, but enough for increasing ASD genetic burden in few generations. This hypothesis could in part be of interest for our work; in fact, even if most of our families are simplex and not multiplex families for ASD, many of them have a familial history of neuropsychiatric disorders.

In addition to these data, we also evidenced, through the analysis of variants segregation, a general equal distribution of paternally and maternally transmitted variants. We found the same situation even considering only extremely rare variants or deleterious variants. This can further support the hypothesis of a threshold effect

model. Moreover, none of the parents analyzed had a diagnosis of ASD, but some of them have a history of or familiarity for other neurodevelopmental disorders (as language delay or hyperactivity/inattention pattern of behavior) or actual psychiatric conditions (as anxiety disorder or mood disorder).

Even if we consider the unique multiplex family included in our study, we found 5 variants for each proband, all inherited, but only 2 shared by the two siblings. Of these variants detected and shared by the probands, one has been already described and reported in literature (Aspromonte et al 2019).

We tried to find a clinical correlation between genetic and familiarity, and we decided to look for significance between genetic burden in our cohort (defined as the total number of variants for each subject) and the familial positive history for Neuropsychiatric condition, but we did not find any correlation; we think that this aspect should be deepen in study with the extension to exome or genome analysis, maintaining a detailed analysis of neuropsychiatric traits in parents and first-degree relatives, adding possibly a clinical screening of the parents.

Another critical point is the type of variants found in our cohort, that are mainly missense variants, that have always been considered of less impact than protein truncating variants. Two reasons might underlie the limited contribution of missense variants. Firstly, the directionality of missense variants does not systematically cause a loss of function; for this reason, to understand the impact of missense mutations researchers require additional information regarding functional annotation. Secondly, the effect size of missense variants is smaller than that of the Protein Truncating Variants; hence, a large sample size is needed for ASD gene discovery (Sanders et al., 2017).

These are some reasons why the role of missense variants in ASD genetic has been previously underestimated, but these works demonstrate that there is growing interest in it.

A recent work of Satterstrom and colleagues (2020), moreover, led light to this point. They conducted an enormous study, including more than 11.000 ASD trios/quartets and they decided to consider not only protein-truncating variants, but also missense variants, dividing them into further groups according to the measures of functional severity.

The proportion of the variance explained by *de novo* missense variants with the highest functional severity prediction was 0.5%, whereas all remaining missense variation explains 0.12%. Thus, in total, all exome *de novo* variants in the autosomes explain 1.92% of the variance of ASD.

This finding underlines the potential role of missense variants, especially for *de novo* ones; however, in an evolving study of ASD understanding, even inherited missense variation could have a role and a contribution in ASD manifestation (Satterstrom et al, 2020).

On this direction goes a WES analysis conducted by An et al. (2014), in which authors identified in average more than a hundred rare (allele frequency ≤ 1%) inherited heterozygous missense variants per ASD subject; they showed significant enrichment of genes previously associated with ASD; moreover, they found that this enrichment was correlated to the rarity of missense variants, defined on the basis of a minor allele frequency or on the functional prediction scores. In addition, they found that rare missense variants inherited from parents with mild symptoms referable to ASD that don't match the diagnostic criteria (the so-called broader autism phenotype, BAP), were significantly enriched for ASD-associated genes compared to variants inherited from parents without BAP. This probably suggests a broad continuum of effect size for rare inherited missense variants. (Choi et al, 2021)

Moreover, the analysis of the 120 genes included in the panel allowed to confirm the role of specific genes, as genetic factors strongly associated to ASD and highlighted a potential stronger involvement of other genes. We noted, in fact, significantly increased frequencies of rare variants in our study population in 6 genes, with more or at least 10 variants each.

In detail, the results indicate that the mutational frequency in all these 6 genes we take into consideration is higher than expected in general population. *FAT1* and *VPS13B* result the most mutated genes in our cohort, with 25 variants detected in *VPS13B* and 19 in *FAT1* gene.

The *FAT1* gene encodes a member of a small family of vertebrate cadherin-like genes whose gene products play a role in cell migration, lamellipodia dynamics, cell polarity, and cell-cell adhesions (summary by Gee et al., 2016). Rare *de novo* missense variants in *FAT1* have been identified in ASD probands by WES in two reports (Neale et al, 2012, Iossifov et al, 2014), while inherited damaging missense variants in *FAT1* have been observed in affected individuals from 3 extended multiplex ASD families (Iossifov et al, 2012). Puppo et al. (2015) identified heterozygous missense variants in *FAT1* in 10 of 49 unrelated Japanese patients with a neuromuscular phenotype similar to facioscapulohumeral muscular dystrophy. Puppo et al. chose FAT1 as a candidate gene based on the findings of Caruso et al. (2013) that demonstrated how hypomorphic *FAT1* mice show an FSHD-like phenotype. Morris et al. (2013) reported recurrent somatic mutations in *FAT1* in glioblastoma, colorectal cancer, and head and neck cancer. In SFARI database *FAT1* is classified as gene with suggestive evidence about its implication in ASD (score 3).

This *VPS13B* gene has been associated with syndromic autism, where a subpopulation of individuals with a given syndrome develop autism. In particular, rare mutations of the *VPS13B* gene were associated to Cohen syndrome

(Kolehmainen et al, 2003). This gene encodes a potential transmembrane protein that may be involved in vesicle-mediated transport and sorting of proteins within the cell. This protein may play a role in the development and the function of the eye, hematological system, and central nervous system. Multiple splice variants encoding distinct isoforms were identified for this gene and when we designed NGS panel *VPS13B* was part of score category S (syndromic). Now *VPS13B* belongs to gene category 1, hence several variants in *VPS13B* have been associated to ASD.

In the whole cohort we found many recurrent variants, and some already described. We found one variant in *SLIT3* gene, already described, in two patients; in one was *de novo,* in the other patient was inherited.
Furthermore, in our cohort we found other 3 variants already described that we found present in 2 patients, and in one case we found a variant co-occurrent in 3 patients. In one case it was found in a couple of siblings, but in the other cases they were found in patients from different Italian regions or even from different countries.
This could be of interest for a particular salience of these variants, as we found them analyzing a little number of patients. Thus, despite they were already associated with ASD, it is difficult to attribute to them a highly pathogenic role, even because there is always more than one variant in other genes.

In the hypothesis of considering variants as susceptibility factors in a familial genetic background, we should not expect to find variants on both alleles of the same gene. Anyway, in our cohort we found one homozygous variant in *MET* gene, inherited from both parents, supporting a potential direct pathogenic role for this mutation. Indeed, *MET* gene is classified as strong candidate on SFARI database (score 2) and positive associations in the Caucasian, Japanese and Italian populations were found

in multiple studies. In addition, biochemical assays showed a reduction in MET protein levels and general disruption of *MET* signaling in ASD patients.

Another point of interest is the presence of significant enrichment in functional networks in some patients with the higher number of variants. We know that this aspect will need functional studies and larger populations to better understand its real value, but this is an important input and an interesting point to deepen in future works.

In a patient, from network functional analysis emerged the enrichment in Histone methyltransferase activity molecular process (GO:0046975), that is involved in epigenetic modification of chromatin and include gene *NSD1* and *ASHL1*. *ASH1L*, a histone methyltransferase, is identified as a top-ranking risk factor for ASD; however, little is known about the biological mechanisms underlying the link of *ASH1L* haploinsufficiency to ASD. A recent work shows that *ASH1L* expression and *H3K4me3* level are significantly decreased in the prefrontal cortex (PFC) of postmortem tissues from ASD patients and revealed the critical role of *ASH1L* in regulating synaptic gene expression and seizures (Qin et al, 2021). Epigenetics, including DNA methylation, represent a powerful gene-environment interaction mechanism and is a topic of interest in ASD pathogenesis study.

As reported in the results, in another patient (A14/06) we found an enrichment that deals with synaptic signaling and neuron synapsis, and even with cellular adhesion. In particular, from our analysis emerge the important role of the Neuroligin family protein binding (*NRXN1* and *NRXN3*) in synapsis maturation and signaling. Even if we found only 3 variants in *NRXN1* and 7 in *NRXN3* in our cohort, these genes seem to emerge as having an important role in critical pathways related to ASD and NDDs. Neurexins are one type of synaptic cell adhesion molecules. They are pre-synaptically localized and bound to neuroligins and other proteins in the post-

synapse. Neurexins and neuroligins, have recently gained more pathological interest as variants in both have been associated with several neuropsychiatric disorders, including autism and schizophrenia. A recent review underlines the strong connection with ASD and their involvement in crucial pathways for synapsis function, sustained by both genetic, functional and animal -model studies (Cuttler et al, 2021).

The result emerged from our study rises interest in the hypothesis of analyzing not only the single gene variants, but also to focus on different variants that could affect a particular functional pathway.

Functional networks are, in fact, an important and deeply studied aspect of neurobiology of ASD, as it is clearer and clearer that the neurobiological basis of many of these disorders, as of all neurodevelopmental disorders, doesn't come from a single gene disease, but from altered mechanisms in many pathways, such as synaptogenesis, neurotransmission and neuronal migration, during the early developmental and/or throughout life (Manoli et al, 2021).

As for phenotypic characterization, we could not find any correlation between genetic burden (express through number of variants per patient) and either to neuropsychologic data nor to familiarity. This could be due to the restricted number of genes included in our panel, or to the pre-selection due to the selection criteria (i.e., the inclusion only of subjects with essential ASD and without instrumental abnormalities). Moreover, we did not find any phenotypic characterization linked to specific genetic variant, and this could be due to the small sample size and to genetic heterogeneity that we found, with different variants coexisting in the same subject, most of them inherited from unaffected parents. Moreover, analyzing these results we are not considering other risk factors that could affect the manifestation of the disorder.

We can therefore hypothesize that a phenotypic classification of ASD subjects could be useful, but we must consider the methods of this phenotypic characterization. Dividing research subjects into groups based only on behavioral aspects may in fact overshadow information about the ways psychopathology gradually emerges across development and about how risk factors operate.

Genetic studies progression unveils the role of shared functional networks, that are not only ASD related, but that seem common to different Neurodevelopment Disorders and Psychiatric conditions. Far from negating the utility of statistical and dimensional diagnostic approach, we think that this aspect opens many other considerations, related to the concept of neurodevelopmental disorders themselves. Considering these issues, the National Institutes of Health (NIH) launched the Research Domain Criteria (RDoC) initiative to develop, for research purposes, new ways of classifying mental disorders based on dimensions of observable behavior and neurobiological measures (Cao et al, 2022).

This should lead, in a long-course perspective, to a deeper comprehension and study of the pathophysiological pathways that are reasonably common to many mental disorders and have finally the aim to identify precise targets for precision medicine. It also suggested that precision medicine in ASDs could not be gene-targeted, but pathway-targeted, as discussed above.

In this perspective the clinical phenotypic characterization should not be a correlation to genetic and functional analysis, but should be a characterization of those findings, as it is already done in many monogenic conditions, and this process must be linked to a wide spectrum of research fields, such as environmental, immunological, neurofunctional and neurostructural studies, and clinical conditions (Neurodevelopmental and Psychiatric conditions).

This study has some important limitations. First of all, the little sample size. We analyzed a sample of 86 patients, but main studies published on this topic have a considerably larger sample size. From the other side, we recruited patients with selected clinical and genetic characteristics, and this is a valuable aspect. Most of the published studies, in fact, include in their samples uncharacterized patients, with complex or essential ASD, and there is not often an instrumental/clinical assessment. The selection of patients without any abnormalities in clinical and instrumental evaluation, and without CNVs, represents from our point of view an important aspect of this work.

Another limitation is due to the lack of a control group, and, for a little part of our sample, the lack of segregation analysis of the variants. We tried to overcome this issue using available genetic database and using them as the population control group.

Future work should be addressed to analyze all members of the affected families (including siblings) and expanding the collection of detailed clinical descriptions to all of them. In particular, we will focus on performing phenotypical characterization of probands' parents and siblings, to detect the presence of any symptoms potentially related to autism and to characterize individuals with clinical or subclinical ASD (BAP). This is crucial to better interpret the contribution of inherited variants. Moreover, we will expand genetic analysis to exome, with the aim of expanding the knowledge about a complex model for ASD genetics. In this direction we think that the effort should be a better understanding and characterization of functional networks. We think it is more and more important to share genetic data and collect larger samples, and together with genetic analysis there should be a shared and careful phenotypic characterization, that should be not an aprioristic

clinical classification, but should help researchers in the clinical characterization of the genetic data.

5. Conclusion

Our study supports the hypothesis that ASD could be the result of a combination of rare deleterious variants (low contribution) and many low-risk alleles (genetic background), as indicated by Huguet and colleagues in 2016. Indeed, most patients in our cohort are carriers of more than one rare variant (with or without *in silico* deleterious prediction) and only 4 patients are carriers of a unique variant.

Looking for a possible genotype-phenotype correlation, we didn't find overlapping phenotypic characteristics among these patients, neither more severe symptoms compared to other patients, as might be expected.

Furthermore, segregation analysis data showed an equal distribution of maternal and paternal inherited variants.

Moreover, recurrence of mutations in a small set of genes, despite the restricted cohort analyzed, demonstrated a clear involvement of these genes in genetic liability to autism.

Finally, we found the involvement of genes related to interesting functional networks involved in synaptic function.

Thus, further studies are needed to deepen our knowledge about the burden of missense variants within functional domains and pathways in ASD.

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8. Appendix

Table 16 - All variants detected

ID patient	Number of variants	GENE	Gene SFARI score	Allele Change	Residue change	SIFT	Poliphen	Genomic coordinate (GRCh37)	Allele frequency (GnomAD)	Philo P Score (conservation score)	Parental transmission	Reference	Allele frequency[1]
H16/18	0	no variants											
E10/24	4	MED12	no rating	c.653C>T	p.Thr218Met	Tolerated	Possibly damaging	X:70340920	0,00015 (1/6500)	3,809	n.a.	Novel	**
E10/24	4	CACNA1C	1	c.3559G>A	p.Val1187Ile	Tolerated	Probably damaging	12:2717819	not present	5,637	n.a.	Novel	***
E10/24	4	DLGAP2	3	c.1086G>A	p.Met362Ile	Deleterious	Probably damaging	8:1513944	0,000004 (1/250000)	3,911	n.a.	Novel	***
E10/24	4	DHCR7	1	c.208G>A	p.Gly70Ser	Tolerated	Benign	11:71155152	0,0004 (1/2500)	0,395	n.a.	Novel	*
O12/11	5	CNTNAP2	2S	c.3950A>G	p.Asn1317Ser	Tolerated	Benign	7:148112662	0,00003 (1/30000)	3,073	Mother	Novel	***
O12/11	5	NRXN1	1	c.2242C>A	p.Leu748Ile	tolerated (0.279)	possibly damaging (0,576)	2:50765412	3,98x10-3 (1/250)	3,101	Mother	Kim HG , et al. (2008)- Onay H , et al. (2017) - Reale et al. (2021)	f
O12/11	5	SOX5	1	c.194A>G	p.Glu65Gly	Tolerated-Low confidence	Benign	12:24048803	not present	3,666	Father	Novel	***
O12/11	5	ANKRD11	1	c.5338G>A	p.Ala1780Thr	tolerated (1)	benign (0)	16:89347612	4,86x10-3 (1/200)	1,302	Father	Novel	f
O12/11	5	CDKL5	1	c.2715C>T	p.905Asp(=)Homo	Synonymous-Splice region variant	Synonymous-Splice region variant	X:18664128	0,00013 (1/7500)	-0,248	Mother	Novel	**
C14/16	2	ASH1L	1	c.6180A>T	p.2060Leu(=)	Synonymous-Splice region variant	Synonymous-Splice region variant	1:155348337	0,00018 (1/5500)	-0,867	n.a.	Novel	**
C14/16	2	VPS13B	1	c.2917A>G	p.Ser973Gly	Tolerated	Possibly damaging	8:100396528	0,00006 (1/16500)	1,601	n.a.	Novel	***
C.P.P	5	SLIT3	no rating	c.4361T>G	p.Val1454Gly	Deleterious	Possibly damaging	5:168093670	0,0004 (1/2500)	4,863	Father	Novel	*
C.P.P	5	DLGAP2	3	c.2314G>A	p.Ala772Thr	Tolerated	Benign	8:1626645	0,0002 (1/5000)	0,108	Mother	Novel	*
C.P.P	5	CACNA1C	1	c.6509_6529delCACAGAGCCCCAATGGCGCCC	p.Pro2170_Ala2176del	/	/	12:2800200	not present	/	Mother	Novel	***
C.P.P	5	RAI1	1	c.1142C>T	p.Ala381Val	deleterious (0.01)	benign (0.184)	17:17697404	0,004 (1/250)	5,344	Mother	Novel	f
C.P.P	5	MED13L	1	c.1189T>C	p.Ser397Pro	Deleterious	Benign	12:116450693	0,00001 (1/100000)	0,698	Father	Novel	***
C.L.	7	MBD5	1	c.236G>A	p.Gly79Glu	Deleterious	Probably damaging	2:149221327	0,0005 (1/2000)	6,358	n.a.	Tolkowski *et al.* 2011	*
C.L.	7	TSC1	1	c.3286T>C	p.Phe1096Leu	Tolerated	Probably damaging	9:135771831	not present	3,212	n.a.	Novel	***
C.L.	7	FAT1	3	c.10220C>T	p.Thr3407Met	Tolerated	Benign	4:187527354	0,00002 (1/50000)	6,048	n.a.	Novel	***
C.L.	7	SLIT3	no rating	c.3268G>A	p.Val1090Met	Tolerated	Benign	5:168114030	0,0002 (1/5000)	0,014	n.a.	Novel	*
C.L.	7	SHANK3	1	c.919G>A	p.Ala307Thr	/	/	22:51121801	not present	3,242	n.a.	Novel	***
C.L.	7	SCN2A	1	c.2723A>G	p.Lys908Arg	tolerated (0.17)	possibly damaging (0.532)	2:166201225	0,004 (1/250)	1,831	n.a.	Novel	f
C.L.	7	ZEB2	no rating	c.2230A>G	p.Ile744Val	tolerated (0.5)	benign (0.046)	2:145156524	0,002 (1/500)	0,306	n.a.	Novel	-
A14/06	6	NRXN1	1	c.163A>G	p.Ser55Gly	Deleterious	Possibly damaging	2:51255249	not present	4,814	Mother	Novel	***
A14/06	6	NRXN3	1	c.2777G>A	p.Arg926His	Deleterious	Benign	14:80164252	0,00005 (1/20000)	6,248	Mother	Novel	***
A14/06	6	ZNF804A	2	c.974G>T	p.Cys325Phe	Tolerated	Probably damaging	2:185801097	0,0007 (1/1400)	0,095	Father	*Novel*	*
A14/06	6	VPS13B	1	c.9424A>G	p.Ser3142Gly	tolerated (0.09)	benign (0.153)	8:100844615	0,009 (1/110)	1,803	Mother	Novel	f
A14/06	6	RELN	1	c.5156C>T	p.Ser1719Leu	deleterious (0)	probably damaging (0.992)	7:103205779	0,006 (1/150)	3,992	Father	Bonora *et al.* 2003	f
A14/06	6	RAI1	1	c.861_872dupGCAGCAGCAGCA	p.Gln288_Gln291dup	/	/	17:17697093	0,003 (1/300)	/	Father	Novel	f

[1] **Allele frequency (GnomAD)< 1/10000 (***), 1/10000<X<1/5000 (**), 1/5000<X<1/1000 (*),1/1000<x<1/500 (-),1/100<x<1/500 (f)**

D.F.M.	1	JARID2	2	c.1523C>T	p.Thr508Met	tolerated (0.37)	benign (0.001)	6:15496979	0,0001 (1/10000)	0,852	Father	Novel	**
E13/58	2	GRIN2B	1	c.190G>A	p.Val64Met	tolerated (0.15)	benign (0.013)	12:14018953	0,00006 (1/15000)	0,238	n.a.	Novel	***
E13/58	2	PCDH9	4	c.3134A>G	p.Tyr1045Cys	tolerated (0.18)	benign (0.123)	13:67477640	0,000004 (1/250000)	0,205	n.a.	Novel	***
A14/55	2	ANKRD11	1	c.890C>T	p.Thr297Met	/	probably damaging (0.985)	16:89352449	1,24x10-3 (1/800)	6,119	*De novo*	Novel	-
A14/55	2	NF1	1	c.1985A>G	p.Lys662Arg	tolerated (0.46)	benign (0.005)	17:29552252	0,00002 (1/50000)	1,096	*De novo*	Novel	***
G14/62	7	DST	3	c.12794C>A	p.Thr4265Asn	deleterious	probably damaging (0.999)	6:56373415	0,000004 (1/250000)	0,637	Father	Novel	***
G14/62	7	DLGAP2	3	c.2915A>T	p.Gln972Leu	deleterious (0)	probably damaging (0.999)	8:1649559	not present	5,027	Mother	Novel	***
G14/62	7	RAI1	1	c.5503G>A	p.Val1835Ile	tolerated (0.16)	benign (0.125)	17:17701765	0,00007 (1/14000)	2,961	Mother	Novel	***
G14/62	7	ZNF804A	2	c.735_737delATT	p.Phe246del	/	/	2:185800857	0,0005 (1/2000)	/	Mother	Novel	*
G14/62	7	ZNF804A	2	c.1490T>C	p.Leu497Pro	deleterious (0.02)	benign (0.011)	2:185801613	0,0004 (1/2500)	-0,299	Mother	Novel	*
G14/62	7	CEP290	3S	c.6401T>C	p.Ile2134Thr	damaging (0.004)	probably damaging (0.999)	12:88454728	6,99x10-3 (1/140)	4,649	Mother	Novel	f
G14/62	7	SYNGAP1	1	c.2710A>G	p.Met904Val	tolerated (0.48)	benign (0.048)	6:33411039	0,000025 (1/40000)	1,823	Father	Novel	***
G.F.M.	2	MED12	no rating	c.3619C>A	p.Leu1207Met	not tolerated	probably damaging (1)	X:70349207	not present	3,243	Mother	Novel	***
G.F.M.	2	DST	3	c.13220A>G	p. Asn4407Ser	tolerated (0.447)	benign (0.016)	6:56357844	0.0009 (1/1100)	1,699	Father	Novel	*
IB1A	5	KIRREL3	3	c.1069G>A	p.Ala357Thr	tolerated (0.12)	probably damaging (0.955)	11:126316710	0,00005 (1/20000)	5,528	Father	Novel	***
IB1A	5	KDM6B	1	c.2701C>T	p.Pro901Ser	/	possibly damaging (0.803)	17:7752307	0,0001 (1/10000)	2,237	Mother	Novel	**
IB1A	5	DMD	S	c.434G>A	p.Arg145Gln	/	possibly damaging (0.498)	X:32834681	0,00003 (1/30000)	5,861	Father	Novel	***
IB1A	5	DHCR7	1	c.1012G>A	p.Val338Met	tolerated (0.27)	benign (0.097)	11:71146837	0,0008 (1/1200)	-1,005	Father	Novel	*
IB1A	5	RAI1	1	c.2709G>C	p.Glu903Asp	deleterious (0.04)	benign (0.383)	17:17698971	0,000008 (1/125000)	-0,072	Father	Novel	***
IB17A	2	NSD1	1	c.2350C>G	p.Gln784Glu	deleterious - low confidence (0.02)	benign (0.043)	5:176637750	0,00002 (1/50000)	2,247	Not maternal	Novel	***
IB17A	2	SCN2A	1	c.2723A>G	p.Lys908Arg	tolerated (0.17)	possibly damaging (0.532)	2:166201225	0,004 (1/250)	1,831	Mother	Novel	f
A15/18	8	ZNF804A	2	c.770G>T	p.Cys257Phe	deleterious (0.05)	probably damaging (0.986)	2:185800893	0,00002 (1/50000)	1,847	n.a.	Novel	***
A15/18	8	VPS13B	1	c.611A>G	p.Asn204Ser	tolerated (0.19)	possibly damaging (0.636)	8:100123356	0,00009 (1/11000)	4,687	n.a.	Novel	***
A15/18	8	VPS13B	1	c.6304C>T	p.Arg2102Cys	tolerated (0.11)	benign (0.025)	8:100711935	0,000008 (1/125000)	0,258	n.a.	Novel	***
A15/18	8	SHANK3	1	c. 4264 C>G	p.Leu1422Val	/	probably damaging (0.998)	22:51160477	0,0001 (1/10000)	1,229	n.a.	Novel	**
A15/18	8	ASH1L	1	c.1823G>A	p.Ser608Asn	tolerated - low confidence (0.13)	benign (0.01)	1:155450838	0,0006 (1/1500)	0,349	n.a.	Novel	*
A15/18	8	SPAST	1	c.875C>G	p.Thr292Ser	tolerated (0.32)	benign (0.008)	2:32340775	not present	2,319	n.a.	Novel	***
A15/18	8	FAT1	3	c.2617G>A	p.Val873Met	/	benign (0.006)	4:187628365	0,0001 (1/10000)	-0,112	n.a.	Novel	**
A15/18	8	AUTS2	1	c.3374_3375insCCA	p.1125insThr	/	/	7:70255576	0,008 (1/125)	/	n.a.	Novel	f
E11/61	4	FAT1	3	c.2641C>T	p.Arg881Cys	/	possibly damaging (0.579)	4:187628341	0,002 (1/500)	0,086	n.a.	Novel	-
E11/61	4	GRIN2A	2	c.2899G>C	p.Val967Leu	tolerated (0.96)	benign (0.005)	16:9858502	0,006 (1/160)	1,125	n.a.	Novel	f
E11/61	4	GNA14	no rating	c.215C>T	p.Thr72Met	tolerated (0.07)	possibly damaging (0.642)	9:80144079	0,0013 (1/750)	3,505	n.a.	Novel	-
E11/61	4	GNA14	no rating	c.97C>T	p.Arg33Cys	deleterious (0.01)	benign (0.195)	9:80262613	0,005 (1/200)	5,939	n.a.	Novel	f
F11/04	3	CACNA1H	2	c.2113C>T	p.Arg705Cys	deleterious (0)	possibly damaging (0.513)	16:1254120	0,00005 (1/20000)	-0,134	Father	Novel	***
F11/04	3	DMD	S	c.9791G>A	p.Arg3264Gln homo	/	benign (0.163)	X:31222094	0,0000056 (1/200000)	5,787	Mother	Novel	***
F11/04	3	GNA14	no rating	c.512C>T	p.Thr171Ile	deleterious (0)	possibly damaging (0.455)	9:80046318	0,003(1/300)	6,039	Father	Novel	f
F12/67	2	FAT1	3	c.9457G>A	p.Asp3153Asn	damaging (0)	probably damaging (0.939)	4:187534269	not present	5,914	Father	Novel	***
F12/67	2	FAT1	3	c.7957G>A	p.Gly2653Ser	deleterious (0)	probably damaging (1)	4:187539783	0,0006 (1/1500)	3,379	Father	Novel	*

D15/81	4	MET	2	c.1988C>T	p.Ser663Leu	tolerated (0.28)	benign (0.198)	7:116397714	0,00002 (1/50000)	1,961	Mother	Novel	***
D15/81	4	SLC6A4	3	c.167G>C	p.Gly56Ala	damaging (0,018)	benign (0)	17:28548810	1,18x10-2 (1/84)	-0,093	Father	Sutcliffe et al. 2005, Adamsen et al. 2010, Reale et al 2021	f
D15/81	4	VPS13B	1	c.2485G>A	p.Ala829Thr	tolerated (0.62)	benign (0)	8:100205255	0,0085 (1/115)	2,199	Father	Novel	f
D15/81	4	CACNA1H	2	c.5113G>A	p.Ala1705Thr	tolerated (0,060)	probably damaging (0,989)	16:1265315	5,51x10-3 (1/180)	5,251	Father	Novel	f
F14/70	5	ASH1L	1	c.5894A>G	p.Glu1965Gly	deleterious - low confidence (0.01)	possibly damaging (0.854)	1:155385649	0,000008 (1/120000)	1,589	Father	Novel	***
F14/70	5	NSD1	1	c.7367T>C	p.Met2456Thr	deleterious - low confidence (0)	benign (0.142)	5:176721736	0,00006 (1/16000)	0,008	Mother	Novel	***
F14/70	5	MET	2	c.3571A>G	p.Thr1191Ala homo	tolerated (0.46)	probably damaging (0.968)	7:116419006	0,000008 (1/120000)	0,859	Father/Mother	Novel	***
F14/70	5	MED13L	1	c.1589C>T	p.Ala530Val	tolerated (0.28)	unknown (0)	12:116446629	0,000007 (1/135000)	5,166	Mother	Novel	***
F14/70	5	POGZ	1	c.4089T>G	p.His1363Gln	tolerated (1)	benign (0)	1:151377422	0,001 (1/1000)	0,687	Mother	Novel	-
M.L. (B)	1	ANK2	1	c.7054_7059delGG TCAA	p.Gly2352_Gln235 3del	/	/	4:114276825	0,000098 (1/11000)	/	n.a.	Novel	***
N13/03	4	TSC2	1	c.446A>G	p.Asn149Ser	deleterious (0.01)	possibly damaging (0.595)	16:2104406	not present	3,038	Father	Novel	***
N13/03	4	CADPS2	3	c.1889T>C	p.Met630Thr	damaging	benign	7:122114544	6,26x10-4 (1/1600)	5,163	Mother	Bonora et al. 2014	*
N13/03	4	DLGAP2	3	c.1751C>T	p.Thr584Met	tolerated (0,125)	benign (0,057)	8:1616675	5,8x10-3 (1/172)	1,839	Father	Novel	f
N13/03	4	CEP290	3S	c.6401T>C	p.Ile2134Thr	damaging (0,004)	probably damaging (0.999)	12:88454728	6,99x10-3 (1/140)	4,649	Mother	Novel	f
L12/26	5	AGAP1	3	c.2479C>T	p.Pro827Ser	tolerated (0.07)	probably damaging (0.988)	2:237032671	0,000087 (1/11000)	3,726	Father	Novel	***
L12/26	5	LAMC3	no rating	c.4534C>A	p.Leu1512Ile	deleterious (0)	probably damaging (0.999)	9:133966980	not present	3,073	Mother	Novel	***
L12/26	5	NRXN1	1	c.1405C>T	p.Pro469Ser	deleterious (0.05)	probably damaging (0.996)	2:50847195	0,004 (1/250)	0,642	Father	Novel	f
L12/26	5	ZNF804A	2	c.538G>A	p.Ala180Thr	tolerated (0.07)	benign (0.027)	2:185800661	0,0008 (1/1200)	0,147	Father	Novel	*
L12/26	5	CACNA1H	2	c.2759C>T	p.Thr920Met	tolerated (1)	benign (0.023)	16:1256259	0,002 (1/500)	2,776	Father	Novel	-
B12/69	4	NSD1	1	c.7367T>C	p.Met2456Thr	deleterious - low confidence (0)	benign (0.142)	5:176721736	0,00006 (1/16000)	0,008	Father	Novel	***
B12/69	4	ANK2	1	c.10901T>A	p.Val3634Asp	/	benign (0.12)	4:114286207	0,002 (1/500)	1,565	Mother	Novel	-
B12/69	4	CSMD1	3	c. 8638G>A	p.Val2880Ile	/	benign (0.003)	8:2832075	0,002 (1/500) Exac	-0,383	Mother	Novel	f
B12/69	4	ASTN2	2	c.2516G>A	p.Arg839Gln	deleterious (0)	unknown (0)	9:119488187	0,0003 (1/3000)	6,303	Mother	Novel	**
D10/14	3	CREBBP	1	c.2941G>A	p.Ala981Thr	/	unknown (0)	16:3819294	0,003 (1/300)	2,848	n.a.	Coupry *et al.* 2002	f
D10/14	3	VPS13B	1	c.11270G>A	p.Arg3757Gln	tolerated (0.1)	possibly damaging (0.553)	8:100874154	0,0017 (1/600)	0,059	n.a.	Novel	-
D10/14	3	VPS13B	1	c.11825_11827dup ATG	p.Asp3942dup	/	/	8:100887648	0,00045 (1/2500)	/	n.a.	Novel	*
C11/79	4	ANK2	1	c.7458C>G	p.His2486Gln	/	benign (0.031)	4:114277232	0,000053	0,202	Father	Novel	***
C11/79	4	CACNA1H	2	c.5996C>T	p.Ser1999Phe	deleterious (0.02)	possibly damaging (0.653)	16:1269078	0,00001 (1/100000)	0,000	Father	Novel	***
C11/79	4	TSC2	1	c.583A>G	p.Ile195Val	tolerated (0.85)	benign (0.019)	16:2105504	0,00004 (1/25000)	0,000	Father	Novel	***
C11/79	4	DMD	S	c.733A>G	p.Ile245Val homo	/	benign (0.005)	X:32717327	0,000045 (1/25000)	0,000	Mother	Novel	***
H11/97	2	RIMS1	1	c.1313C>T	p.Ala438Val	tolerated (0.63)	benign (0.003)	6:72892487	not present	0,000	n.a.	Novel	***
H11/97	2	FAT1	3	c.4841C>T	p.Pro1614Leu	damaging (0,007)	possibly damaging (0.903)	4:187542899	0,003 (1/300)	0,000	n.a.	Novel	f
C15/87	5	EHMT1	1	c.239A>T	p.Asn80Ile	deleterious - low confidence (0.01)	benign (0.001)	9:140611231	0,00002 (1/50000)	1,248	Mother	Novel	***
C15/87	5	FAT1	3	c.6782C>T	p.Thr2261Met	damaging (0,022)	probably damaging (0.995)	4:187540958	0,0008 (1/1200)	4,227	Father	Novel	*
C15/87	5	DLGAP2	3	c.1852G>A	p.Asp618Asn	tolerated (0.17)	probably damaging (0.981)	8:1616776	0,0008 (1/1200)	4,458	Mother	Novel	*
C15/87	5	CSMD1	3	c.4220G>C	p.Arg1407Thr	tolerated	possibly damaging (0.709)	8:3087687	0,006 (1/160) ExAC	4,235	Mother	Novel	f
C15/87	5	NLGN3	1	c.10C>T	p.Arg4Trp homo (chr X)	deleterious - low confidence (0.04)	benign (0.066)	X:70367609	0,0007 (1/1500)	-0,095	Mother	Novel	*

C14/83	4	SLC9A9	3	c.1844C>T	p.Thr615Met	tolerated - low confidence (0.1)	benign (0.366)	3:142985638	0,00004 (1/25000)	1,857	n.a.	Novel	***
C14/83	4	DST	3	c.13388 C>T	p.Thr4463Ile	tolerated (0.069)	probably damaging (0.974)	6:56357176	0,00002 (1/50000) ExAC	4,342	n.a.	Novel	***
C14/83	4	VPS13B	1	c.9667C>T	p.Arg3223Trp	deleterious (0.02)	possibly damaging (0.689)	8:100844858	0,004 (1/250)	0,097	n.a.	Novel	f
C14/83	4	ANKRD11	1	c.5578C>T	p.Pro1860Ser	tolerated (0,098)	probably damaging (0.998)	16:89347372	0,00053 (1/2000)	2,106	n.a.	Novel	*
M14/99	2	FAT1	3	c.4841C>T	p.Pro1614Leu	damaging (0,007)	possibly damaging (0.903)	4:187542899	0,003 (1/300)	6,015	n.a.	Novel	f
M14/99	2	EHMT1	1	c.905A>G	p.Lys302Arg	deleterious - low confidence (0)	probably damaging (0.993)	9:140637904	0,0002 (1/5000)	3,892	n.a.	Novel	**
L14/95	5	SLIT3	no rating	c.1886G>A	p.Ser629Asn	tolerated (0.13)	benign (0.073)	5:168180047	0,007 (1/140)	4,356	*De novo*	Cukier *et al.* 2014	f
L14/95	5	MBD5	1	c.2030G>A	p.Ser677Asn	tolerated	benign (0)	2:149227542	2,23x10-3 (1/450)	2,434	Mother	Novel	f
L14/95	5	ANK2	1	c.2472_2474delCAC	p.Thr826del	/	/	4:114214678	Exac 0,0007 (1/1500)	/	Mother	Novel	*
L14/95	5	VPS13B	1	c.8978A>G	p.Asn2993Ser	deleterious (0.02)	benign (0.292)	8:100832259	0,003 (1/300)	5,257	Mother	Novel	f
L14/95	5	KDM6B	1	c.3940C>T	p.Pro1314Ser	tolerated	benign (0.14)	17:7754708	0,0002 (1/5000)	2,646	Mother	Novel	**
s.s.73499	1	FAT1	3	c.2563G>A	p.Gly855Arg	damaging (0,034)	probably damaging (1)	4:187628419	2,11x10-3 (1/470)	5,809	Father	Novel	f
r.g.74126	5	FAT1	3	c.12653 A>G	p.Asp4218Gly	tolerated	probably damaging	4:187518041	1,02x10-2 (1/98)	4,958	Mother	Cukier *et al.* 2014	f
r.g.74126	5	ZEB2	no rating	c.2230A>G	p.Ile744Val	tolerated (0.5)	benign (0.046)	2:145156524	0,002 (1/500)	0,306	Father	Novel	-
r.g.74126	5	CSMD1	3	c.8935G>A	p.Gly2979Ser	damaging (0,005)	probably damaging (1)	8:2824257	5,45x10-3 (1/183)	6,072	Mother	Novel	f
r.g.74126	5	AVPR1A	2	c.1193C>G	p.Thr398Arg	tolerated (0.64)	possibly damaging (0.699)	12:63541203	0,0009 (1/1100)	0,181	Mother	Novel	*
r.g.74126	5	RAI1	1	c.1142C>T	p.Ala381Val	deleterious (0.01)	benign (0.184)	17:17697404	0,004 (1/250)	5,344	Father	Novel	f
a.d.81815	5	FAT1	3	c.9845C>T	p.Ser3282Phe	damaging	possibly damaging (0.781)	4:187532548	0,000004 (1/250000)	4,268	Mother	Novel	***
a.d.81815	5	CSMD1	3	c.4375G>A	p.Ala1459Thr	conflicting	benign (0.034)	8:3081360	0,00006 (1/16000) ExAC	1,266	Father	Novel	***
a.d.81815	5	ANKRD11	1	c.2759G>A	p.Arg920Lys	tolerated (0,95)	benign (0.054)	16:89350191	0,00001 (1/100000)	1,995	Mother	Novel	***
a.d.81815	5	ZEB2	no rating	c.2230A>G	p.Ile744Val	tolerated (0.5)	benign (0.046)	2:145156524	0,002 (1/500)	0,306	Mother	Novel	-
a.d.81815	5	RAI1	1	c.1142C>T	p.Ala381Val	deleterious (0.01)	benign (0.184)	17:17697404	0,004 (1/250)	5,344	Father	Novel	f
g.w.g.01350	2	FOXP2	3	c.967T>G	p.Ser323Ala	tolerated (0.21)	unknown (0)	7:114282581	0,00002 (1/50000)	4,498	n.a.	Novel	***
g.w.g.01350	2	CSMD1	3	c.1129G>T	p.Asp377Tyr	/	probably damaging (1)	8:3443751	0,000009 (1/110000) ExAC	6,224	n.a.	Novel	***
p.a.59939	4	ANK2	1	c.9424A>C	p.Thr3142Pro	/	possibly damaging (0.522)	4:114279198	0,0001 (1/10000)	0,156	Father	Novel	**
p.a.59939	4	AGAP1	3	c.2393G>A	p.Arg798Gln	tolerated (0.07)	probably damaging (0.998)	2:237032585	0,00034 (1/3000)	0,545	Mother	Novel	**
p.a.59939	4	VPS13B	1	c.1981G>A	p.Asp661Asn	deleterious (0.03)	benign (0.009)	8:100160206	0,0004 (1/2500)	1,831	Mother	Novel	*
p.a.59939	4	CACNA1A	1S	c.3040G>A	p.Glu1014Lys	/	benign (0.035)	19:13409407	0,003 (1/300)	4,101	Father	Novel	f
o.f.76867	2	ANK2	1	c.11584C>T	p.Leu3862Phe	/	benign (0.003)	4:114290935	0,0000041 (1/244000)	-0,009	Father	Novel	***
o.f.76867	2	ST7	3	c.1700G>T	p.Arg567Leu	deleterious - low confidence (0.02)	benign (0.02)	7:116862976	0,006 (1/160)	0,048	Father	Novel	f
H.S.M.	4	TSC1	1	c.1960 C>G	p.Gln654Glu	tolerated (0.07)	benign (0.015)	9:135781005	7,9x10-4 (1/1200)	2,597	Mother	Kelleher *et al.* 2012	*
H.S.M.	4	LAMC3	no rating	c.4396G>A	p.Glu1466Lys	tolerated (0.38)	benign (0.004)	9:133963123	0,0002 (1/5000)	-0,985	Mother	Novel	**
H.S.M.	4	ANKRD11	1	c.280G>C	p.Ala94Pro	/	benign (0.247)	16:89357538	0,0004 (1/2500)	0,282	Father	Novel	*
H.S.M.	4	KATNAL2	1	c.577T>G	p.Leu193Val	deleterious (0)	possibly damaging (0.831)	18:44593458	0,001 (1/1000)	0,595	Father	Novel	-
LDM160	4	VPS13B	1	c.2116A>T	p.Met706Val	deleterious (0)	benign (0.267)	8:100168879	0,00002 (1/50000)	4,488	Mother	Novel	***
LDM160	4	SHANK3	1	c.845C>T	p.Ser282Leu	/	/	22:51117816	0,000008 (1/120000)	5,041	Father	Novel	***
LDM160	4	RELN	1	c.1483A>G	p.Ile495Val	tolerated (0.37)	benign (0.014)	7:103294611	0,0002 (1/5000)	0,013	Mother	Novel	**
LDM160	4	DMD	S	c.7892G>A	p.Arg2631His	/	possibly damaging (0.715)	X:31676242	0,00002 (1/50000)	1,347	Mother	Novel	***
LDM167	4	PTEN	1	c.1131_1132dupTA	p.Arg378IlefsTer39	/	/	10:89725145	not present	/	*De novo*	Novel	***
LDM167	4	CREBBP	1	c.2697_2708delCACCCCGACTCC	p.Thr900_Pro903del	/	/	16:3820742	not present	/	Father	Novel	***

LDM167	4	FAT1	3	c.7700G>A	p.Arg2567His	/	possibly damaging (0.873)	4:187540040	0,008 (1/120)	3,064	Father	Novel	f
LDM167	4	VPS13B	1	c.2485G>A	p.Ala829Thr	tolerated (0.62)	benign (0)	8:100205255	0,0085 (1/115)	2,199	Mother	Novel	f
H14/09 (B.F.)	4	MBD5	1	c.3280G>A	p.Asp1094Asn	deleterious - low confidence (0.01)	unknown (0)	2:149247180	1,19x10-5 (1/84000)	5,549	Mother	Novel	***
H14/09 (B.F.)	4	NSD1	1	c.461A>T	p.Glu154Val	deleterious - low confidence (0.01)	possibly damaging (0.478)	5:176562565	not present	2,767	Father	Novel	***
H14/09 (B.F.)	4	DST	3	c.5492T>C	p.Leu1831Ser	damaging (0.001)	probably damaging (1)	6:56437738	2,91x10-4 (1/3400)	4,808	Father	Novel	*
H14/09 (B.F.)	4	AUTS2	1	c.3635C>T	p.Pro1212Leu	deleterious (0.03)	probably damaging (0.999)	7:70255837	8,21x10-6 (1/121000)	5,353	Mother	Novel	***
P.A.-79782	5	RELN	1	c.3210G>T	p.Met1070Ile	tolerated (0.13)	benign (0.003)	7:103243874	6,4x10-5 (1/15625)	2,584	Mother	Novel	***
P.A.-79782	5	VPS13B	1	c.1832G>A	p.Arg611Lys	tolerated (0.92)	benign (0.307)	8:100155382	1,27x10-3 (1/780)	0,217	Mother	Ionita-Laza I , et al. (2014)	*
P.A.-79782	5	GNA14	no rating	c.592C>T	p.Arg198Trp	deleterious (0)	possibly damaging (0.793)	9:80046238	7,8x10-5 (1/12800)	2,644	Father	Novel	***
P.A.-79782	5	NRXN3	1	c.127C>T	p.Arg43Cys	deleterious (0)	probably damaging (0.981)	14:79175584	4x10-5 (1/25000)	3,193	Mother	Novel	***
P.A.-79782	5	ANKRD11	1	c.890C>T	p.Thr297Met	/	probably damaging (0.985)	16:89352449	1,24x10-3 (1/800)	6,119	Mother	Novel	-
C.M.L.-63035	3	VPS13B	1	c.1639A>G	p.Thr547Ala	tolerated	benign (0)	8:100148968	$2,26x10^{-4}$ (1/4400)	1,083	Father	Novel	*
C.M.L.-63035	3	ANKRD11	1	c.4546G>A	p.Asp1516Asn		benign (0.357)	16:89348404	not present	3,113	Father	Novel	***
C.M.L.-63035	3	SLC6A4	3	c.577G>A	p.Asp193Asn	tolerated (0.4)	benign (0.003)	17:28545257	$1,73x10^{-4}$ (1/5700)	0,327	Father	Novel	**
P.J.A.-84366	6	NTNG1	3S	c.1617C>G	p.Phe539Leu	deleterious - low confidence (0.01)	unknown (0)	1:108023459	1,71x10-4 (1/5800)	0,898	Mother	Novel	**
P.J.A.-84366	6	AGAP1	3	c.2503G>A	p.Ala835Thr	tolerated (0.33)	probably damaging (0.991)	2:237032695	not present	3,943	Father	Novel	***
P.J.A.-84366	6	DST	3	c.11314C>G	p.Gln3772Glu	/	benign (0.087)	6:56393648	6,8x10-5 (1/14700)	5,956	Mother	Novel	***
P.J.A.-84366	6	TSC1	1	c.1960C>G	p.Gln654Glu	tolerated (0.07)	benign (0.015)	9:135781005	7,9x10-4 (1/1200)	2,597	Mother	Kelleher *et al.* 2012	*
P.J.A.-84366	6	PTPN11	1	c.392A>G	p.Lys131Arg	tolerated (0.29)	probably damaging (0.99)	12:112891058	5,3x10-5 (1/18800)	3,535	Mother	Novel	***
P.J.A.-84366	6	ATRX	1	c.1825C>G	p.Pro609Ala	/	benign (0.011)	X:76938923	1x10-3 (1/1000)	-0,073	Father	Brett M , et al. (2014)	-
M.S.-64656	12	POGZ	1	c.2669C>T	p.Ala890Val	tolerated (0.43)	benign (0)	1:151378842	$6x10^{-4}$ (1/1600)	-0,509	Mother	Novel	*
M.S.-64656	12	SATB2	3S	c.1850C>T	p.Ser617Phe	deleterious (0.01)	benign (0.188)	2:200137286	$7,96x10^{-6}$ (1/125000)	3,084	Father	Novel	***
M.S.-64656	12	ANK2	1	c.6854T>C	p.Ile2285Thr	/	benign (0.003)	4:114276628	$1,25x10^{-4}$ (1/8000)	-0,499	Mother	Novel	**
M.S.-64656	12	ANK2	1	c.11716C>T	p.Arg3906Trp	/	probably damaging (1)	4:114294462	$1,07x10^{-3}$ (1/900)	2,041	Mother	Novel	-
M.S.-64656	12	NIPBL	1	c.5690A>G	p.Asn1897Ser	tolerated (0.06)	probably damaging (0.991)	5:37024802	$7,09x10^{-5}$ (1/14000)	4,743	Father	Novel	***
M.S.-64656	12	RELN	1	c.7438G>A	p.Gly2480Ser	tolerated (0.35)	probably damaging (1)	7:103163890	$2,62x10^{-3}$ (1/380)	4,089	Mother	Bonora et al. 2003	f
M.S.-64656	12	CSMD1	3	c.7688A>T	p.Gln2563Leu	benign (0.001)	/	8:2887008	$8,61x10^{-6}$ (1/116000)	1,082	Father	Novel	***
M.S.-64656	12	CEP290	3S	c.5998A>G	p.Ile2000Val	/	benign (0.008)	12:88465084	$1,72x10^{-4}$ (1/1300)	1,279	Father	Novel	*
M.S.-64656	12	CEP290	3S	c.1092T>G	p.Ile364Met	/	probably damaging (0.997)	12:88519120	$8,44x10^{-4}$ (1/1100)	1,595	Father	Novel	*
M.S.-64656	12	CEP290	3S	c.963T>A	p.Asp321Glu	/	probably damaging (0.998)	12:88520195	$9,13x10^{-5}$ (1/10100)	1,709	Father	Novel	***
M.S.-64656	12	ANKRD11	1	c.5088C>G	p.Asp1696Glu	/	benign (0.015)	16:89347862	$8x10^{-4}$ (1/1250)	0,186	Mother	Novel	*
M.S.-64656	12	RAI1	1	c.3781_3783delGAG	p.Glu1261del	/	/	17:17700037	2,82x10-3 (1/350)	/	Mother	Novel	f
M.A.-50264	3	SYNGAP1	1	c.3858A>T	p.Glu1286Asp	tolerated (1)	benign (0.007)	6:33415683	7x10-5 (1/14200)	-0,051	Mother	Novel	***
M.A.-50264	3	MED13L	1	c.3512A>G	p.Lys1171Arg	tolerated (1)	unknown (0)	12:116429247	3,2x10-3 (1/300)	1,697	Father	Novel	f
M.A.-50264	3	CHD8	1	c.4385G>A	p.Arg1462Gln	tolerated (0.08)	benign (0.194)	14:21868757	9,4x10-6 (1/106000)	2,662	Father	Novel	***
A.A.A.K.-86859	4	DST	3	c.7549G>A	p.Asp2517Asn	/	probably damaging (0.967)	6:56425092	4,91x10-4 (1/2000)	6,038	Father	Novel	*
A.A.A.K.-86859	4	CADPS2	3	c.32C>T	p.Ser11Leu	damaging (0.0011)	/	7:122526360	2,21x10-4 (1/4500)	2,376	Father	Novel	*
A.A.A.K.-86859	4	MED13L	1	c.4522C>T	p.His1508Tyr	tolerated (0.67)	unknown (0)	12:116421994	4,04x10-4 (1/2400)	0,399	Mother	Novel	*
A.A.A.K.-86859	4	SMG6	3	c.2392C>T	p.Pro798Ser	deleterious (0)	probably damaging (1)	17:2186975	4,77x10-5 (1/20900)	5,829	Father	Novel	***

C17/76	5	CNTN4	2	c.1805C>A	p.Ala602Asp	tolerated (0.8)	benign (0.006)	3:3076337	6,37x10-5 (1/15700)	1,211	Mother[2]	Novel	***
C17/76	5	DLGAP2	3	c.1751C>T	p.Thr584Met	tolerated (0,125)	benign (0,057)	8:1616675	5,8x10-3 (1/172)	1,839	Mother[2]	Novel	f
C17/76	5	EHMT1	1	c.2093C>T	p.Pro698Leu	tolerated (0.87)	benign (0.002)	9:140672408	7,96x10-6 (1/125000)	0,144	Father[2]	Novel	***
C17/76	5	PTEN	1	c.235G>A	p.Ala79Thr	tolerated (0.59)	benign (0.005)	10:89690828	1,03x10-4 (1/9700)	3,056	Father[2]	Aspromonte MC , et al. (2019)	**
C17/76	5	CACNA1H	2	c.4685G>A	p.Arg1562Gln	deleterious (0.01)	probably damaging (0.99)	16:1262064	1,21x10-5 (1/82600)	5,306	Mother[2]	Novel	***
LDM568	5	DLGAP2	3	c.2643G>T	p.Met881Ile	tolerated (0.11)	benign (0.004)	8:1645399	1,79x10-5 (1/55800)	1,664	Father[2]	Novel	***
LDM568	5	PTEN	1	c.235G>A	p.Ala79Thr	tolerated (0.59)	benign (0.005)	10:89690828	1,03x10-4 (1/9700)	3,056	Father[2]	Aspromonte MC , et al. (2019)	**
LDM568	5	CACNA1H	2	c.4685G>A	p.Arg1562Gln	deleterious (0.01)	probably damaging (0.99)	16:1262064	1,21x10-5 (1/82600)	5,306	Mother[2]	Novel	***
LDM568	5	KATNAL2	1	c.617A>C	p.Gln206Pro	deleterious (0)	probably damaging (0.939)	18:44595598	2x10-5 (1/50000)	3,553	Mother[2]	Novel	***
LDM568	5	MED12	no rating	c.5711C>T	p.Ala1904Val	/	benign (0.102)	X:70357196	1,11x10-3 (1/900)	1,888	Father[2]	Novel	-
D.G.M.-50685	5	SLIT3	no rating	c.358A>G	p.Lys120Glu	tolerated (0.19)	possibly damaging (0.716)	5:168620538	7,08x10-5 (1/14100)	3,144	Father	Novel	***
D.G.M.-50685	5	RELN	1	c.8798C>T	p.Thr2933Ile	deleterious (0.01)	possibly damaging (0.493)	7:103138569	2,02x10-4 (1/4900)	2,648	Mother	Novel	*
D.G.M.-50685	5	CEP290	3S	c.1079G>A	p.Arg360Gln	/	possibly damaging (0.861)	12:88519133	5,15x10-3 (1/190)	0,474	Father	Deciphering Developmental Disorders Study (2014)	f
D.G.M.-50685	5	PCDH9	3	c.2714G>C	p.Ser905Thr	tolerated (0,065)	possibly damaging (0,873)	13:67799859	not present	4,105	Father	Novel	***
D.G.M.-50685	5	KDM6B	1	c.1913C>G	p.Pro638Arg	Damaging (0)	unknown (0)	17:7751519	4,13x10-6 (1/24200)	0,858	Mother	Novel	***
D16/37	2	KDM6B	1	c.791_792insACCCCC	p.Pro263_Pro264dup	/	/	17:7750214	4,6x10-5 (1/21700)	/	Mother	Novel	***
D16/37	2	CACNA1A	1S	c.1357G>A	p.Ala453Thr	/	probably damaging (0.998)	19:13428124	4,65x10-3 (1/215)	5,448	Mother	Novel	f
LDM565	2	LAMC3	no rating	c.3188A>G	p.Tyr1063Cys	deleterious (0.02)	possibly damaging (0.827)	9:133946989	1,24x10-4 (1/8000)	2,644	Father	Novel	**
LDM565	2	MED12	no rating	c.5711C>T	p.Ala1904Val	/	benign (0.102)	X:70357196	1,11x10-3 (1/900)	1,888	Mother	Novel	-
LDM632	6	SLIT3	no rating	c.460C>T	p.Arg154Cys	deleterious (0)	probably damaging (0.998)	5:168310295	2,72x10-4 (1/3600)	2,469	Mother	Novel	*
LDM632	6	GRIN2A	2	c.4307A>G	p.Asn1436Ser	tolerated (0.8)	benign (0.011)	16:9857094	6,33x10-4 (1/1500)	2,222	Mother	Novel	*
LDM632	6	VPS13B	1	c.7753G>A	p.Glu2585Lys	tolerated (0,386)	benign (0,020)	8:100791158	3,15x10-3 (1/300)	1,788	Father	Novel	f
LDM632	6	NSD1	1	c.2339C>T	p.Ser780Leu	tolerated - low confidence (1)	benign (0.001)	5:176637739	1,1x10-3 (1/900)	0,293	Mother	Novel	-
LDM632	6	DYRK1A	1	c.1066A>G	p.Thr356Ala	deleterious (0)	probably damaging (0.95)	21:38865433	2,03x10-3 (1/490)	5,205	Father	Novel	f
LDM632	6	PTCHD1	1	c.97G>A	p.Val33Met	tolerated (0.06)	benign (0.131)	X:23353089	not present	0,259	Mother	Novel	***
LDM695	1	BRAF	1	c.1180T>C	p.Ser394Pro	tolerated (0.11)	benign (0.424)	7:140482955	6,37x10-5 (1/15700)	4,824	Father	Novel	***
51432	2	POGZ	1	c.4166A>G	p.Glu1389Gly	deleterious (0)	probably damaging (0.993)	1:151377345	not present	4,114	Father	Novel	***
51432	2	CEP290	3S	c.4037A>G	p.Asn1346Ser	/	benign (0.001)	12:88481714	not present	-0,472	Mother	Novel	***
73206	8	MBD5	1	c.692T>C	p.Ile231Thr	deleterious (0.01)	benign (0.291)	2:149226204	6,03x10-5 (1/16500)	3,403	Mother	Novel	***
73206	8	SCN1A	1	c.1811G>A	p.Arg604His	tolerated (0.32)	probably damaging (1)	2:166900411	1,38x10-3 (1/700)	6,022	Mother	Alvarez-Mora MI, et al. (2016)	-
73206	8	JARID2	2	c.977G>T	p.Arg326Leu	deleterious - low confidence (0.01)	possibly damaging (0.669)	6:15496433	2,39x10-5 (1/41000)	3,535	Mother	Novel	***
73206	8	STXBP5	2	c.3374T>C	p.Met1125Thr	deleterious (0)	possibly damaging (0.87)	6:147704094	not present	4,653	Father	Novel	***
73206	8	SHANK2	1	c.2188G>A	p.Arg730Thr	tolerated (0.134)	/	11:70348913	6,79x10-5 (1/14700)	2,889	Mother	Novel	***
73206	8	TSC2	1	c.2584G>A	p.Ala862Thr	deleterious (0.01)	possibly damaging (0.466)	16:2125838	3,9x10-5 (1/25600)	5,827	Mother	Novel	***

[2] Multiplex family

73206	8	ANKRD11	1	c.5338G>A	p.Ala1780Thr	tolerated (1)	benign (0)	16:89347612	4,86x10-3 (1/200)	1,302	Father	Novel	f
73206	8	DMD	S	c.821A>G	p.Tyr274Cys	/	probably damaging (0.974)	X:32717239	1,22x10-4 (1/8100)	1,598	Mother	Novel	**
73400	4	SCN2A	1	c.2723A>G	p.Lys908Arg	tolerated (0.17)	possibly damaging (0.532)	2:166201225	4,21x10-3 (1/230)	1,831	Father	Novel	f
73400	4	RELN	1	c.854T>C	p.Leu285Ser	tolerated (1)	benign (0)	7:103341405	3,98x10-6 (1/251000)	3,162	Mother	Novel	***
73400	4	CSMD1	3	c.2073A>C	c.2073A>C(p.=)	tolerated (1)	/	8:3263742	5,8x10-5 (1/17200)	-0,199	Father	Novel	***
73400	4	KDM6B	1	c.1237G>A	p.Val413Met	tolerated (0.113)	unknown (0)	17:7750750	3,98x10-5 (1/25100)	-0,498	Mother	Novel	***
75544	6	ASH1L	1	c.7568G>A	p.Arg2523His	deleterious - low confidence (0)	probably damaging (0.966)	1:155317682	2,11x10-3 (1/470)	5,566	Father	Novel	f
75544	6	VPS13B	1	c.3386A>G	p.Lys1129Arg	deleterious (0.01)	benign (0.001)	8:100454804	4,61x10-3 (1/200)	2,125	Mother	Novel	f
75544	6	CACNA1C	1	c.5294C>G	p.Ala1765Gly	tolerated (0.051)	/	12:2788668	7,86x10-4 (1/1200)	2,652	Mother	Novel	*
75544	6	CACNA1H	2	c.3175G>T	p.Ala1059Ser	tolerated (0.16)	benign (0.26)	16:1258033	6,97x10-3 (1/140)	1,786	Father	Novel	f
75544	6	TSC2	1	c.5376G>C	p.Gln1792His	deleterious - low confidence (0)	probably damaging (0.998)	16:2138563	not present	2,246	Mother	Novel	***
75544	6	ANKRD11	1	c.5338G>A	p.Ala1780Thr	tolerated (1)	benign (0)	16:89347612	4,86x10-3 (1/200)	1,302	Mother	Novel	f
52984	3	MBD5	1	c.2030G>A	p.Ser677Asn	tolerated	benign (0)	2:149227542	2,23x10-3 (1/450)	2,434	Father	Novel	f
52984	3	SCN1A	1	c.133G>A	p.Asp45Asn	tolerated (0.1)	benign (0.109)	2:166929999	7,95x10-6 (1/125000)	2,135	Father	Carvill GL , et al. (2013)	***
52984	3	NF1	1	c.5026G>A	p.Ala1676Thr	tolerated (0.1)	benign (0.271)	17:29653028	7,95x10-6 (1/125000)	3,174	Father	Novel	***
65706	7	FAT1	3	c.8852A>G	p.Asn2951Ser	/	probably damaging (0.999)	4:187538888	1,35x10-3 (1/740)	4,884	Father	Novel	-
65706	7	SLIT3	no rating	c.1421G>A	p.Arg474His	deleterious (0)	probably damaging (0.996)	5:168199824	1,42x10-5 (1/70400)	6,086	Father	Novel	***
65706	7	AUTS2	1	c.3548G>A	p.Ser1183Asn	deleterious (0.03)	benign (0.075)	7:70255750	6,24x10-4 (1/1600)	3,396	Father	Novel	*
65706	7	VPS13B	1	c.9110C>G	p.Thr3037Ser	tolerated (0.17)	benign (0.292)	8:100833562	1,31x10-4 (1/7600)	0,568	Mother	Novel	**
65706	7	SHANK2	1	c.901G>A	p.Glu301Lys	damaging (0.002)	/	11:70803479	not present	5,506	Mother	Novel	***
65706	7	CACNA1H	2	c.3283G>A	p.Asp1095Asn	deleterious (0.04)	probably damaging (0.959)	16:1258141	3,33x10-4 (1/3000)	4,327	Father	Novel	**
65706	7	ANKRD11	1	c.3431C>A	p.Pro1144Gln	tolerated (0,731)	benign (0.001)	16:89349519	1,06x10-5 (1/94000)	-0,455	Father	Novel	***
H16/06	2	VPS13B	1	c.5980A>G	p.Ile1994Val	tolerated (0.58)	benign (0)	8:100654723	1,58x10-3 (1/630)	0,258	Mother	Novel	-
H16/06	2	NRXN3	1	c.3156G>T	p.Gln1052His	damaging (0,014)	benign (0,252)	14:80328277	7,85x10-5 (1/12700)	6,289	Father	Novel	***
E17/07	3	FAT1	3	c.3749A>G	p.Tyr1250Cys	/	probably damaging (0.99)	4:187557962	2x10-3 (1/500)	5,032	Father	Novel	f
E17/07	3	DST	3	c.9604A>T	p.Ser3202Cys	/	benign (0.018)	6:56417095	1,09x10-3 (1/900)	0,452	Father	Novel	-
E17/07	3	MED13L	1	c.3801T>A	p.Asp1267Glu	tolerated (1)	unknown (0)	12:116428958	1,19x10-5 (1/84000)	0,019	Father	Novel	***
G16/59	0	no variants	/	/	/	/	/	/	/	/	/	/	/
G17/11	5	NRXN1	1	c.1873C>T	p.Arg625Trp	deleterious (0)	probably damaging (0.937)	2:50779731	3,19x10-5 (1/31300)	2,206	Father	Novel	***
G17/11	5	GNA14	no rating	c.497C>A	p.Pro166Gln	tolerated (0.08)	benign (0.412)	9:80046333	not present	6,039	Mother	Novel	***
G17/11	5	CACNA1C	1	c.5788T>C	p.Ser1930Pro	tolerated (0.143)	/	12:2794972	1,43x10-4 (1/6900)	3,459	Father	Novel	**
G17/11	5	CACNA1H	2	c.6322G>A	p.Ala2108Thr	tolerated (0.08)	benign (0.359)	16:1270254	1,42x10-3 (1/700)	4,825	Father	Chourasia N , et al. (2019)	-
G17/11	5	CREBBP	1	c.7162G>A	p.Ala2388Thr	tolerated (0.156)	/	16:3777886	1,99x10-5 (1/50200)	4,206	Mother	Novel	***
75323	4	HOXA1	S	c.194A>C	p.His65Pro	tolerated	benign (0,277)	7:27135338	4,4x10-4 (1/2200)	0,173	mother	Novel	*
75323	4	GABRB3	1	c.31C>T	p.Pro11Ser	tolerated (0,703)	benign (0,137)	15:27018841	2,95x10-3 (1/330)	1,308	father	Delahanty RJ, et al. (2009) e Alvarez-Mora MI, et al. (2016)	f
75323	4	SMG6	3	c.835C>T	p.Arg279Cys	damaging (0,032)	probably damaging (0,999)	17:2203212	6,37x10-5 (1/15700)	1,073	mother	Novel	***
75323	4	SLC6A4	3	c.167G>C	p.Gly56Ala	damaging (0,018)	benign (0)	17:28548810	1,18x10-2 (1/84)	-0,093	father	Sutcliffe et al. 2005, Adamsen et al. 2010, Reale et al 2021	f
LDM733	6	GRIN2A	2	c.422C>T	p.Thr141Met	tolerated (0,206)	benign (0.022)	16:10032401	1,1x10-3 (1/900)	0,825	mother	Novel	-
LDM733	6	LAMC3	no rating	c.1688G>A	p.Arg563Gln	tolerated (0,275)	benign (0,066)	9:133927935	1,3x10-4 (1/7600)	-0,572	mother	Novel	**
LDM733	6	LAMC3	no rating	c.4415G>A	p.Arg1472Gln	tolerated (0,199)	benign (0,016)	9:133963142	3,5x10-4 (1/2800)	-0,023	father	Novel	*
LDM733	6	KDM6B	1	c.625G>T	p.Val209Leu	damaging (0,031)	benign (0)	17:7749972	4,3x10-3 (1/230)	0,155	father	Novel	f

LDM733	6	FAT1	3	c.12653A>G	p.Asp4218Gly	tolerated	probably damaging	4:187518041	1,02x10-2 (1/98)	4,958	father	Cukier et al. 2014	f
LDM733	6	FAT1	3	c.4358G>A	p.Arg1453His	damaging (0,018)	probably damaging (1)	4:187549883	1,5x10-3 (1/660)	4,467	father	Novel	-
52724	2	VPS13B	1	c.1559A>G	p.His520Arg	deleterious	probably damaging	8:100147957	5,81x10-4 (1/1700)	4,421	mother	Ionita-Laza et al., 2014	*
52724	2	GRM5	3	c.2095A>C	p.Ile699Leu	tolerated	benign	11:88300756	1,42x10-5 (1/70400)	0,434	father	Novel	***
70102	6	MBD5	1	c.2162C>T	p.Pro721Leu	damaging	benign	2:149227674	1,63x10-4 (1/6000)	2,955	father	Novel	**
70102	6	FAT1	3	c.12653A>G	p.Asp4218Gly	tolerated	probably damaging	4:187518041	1,02x10-2 (1/98)	4,958	father	Cukier et al. 2014	f
70102	6	STXBP5	2	c.1273C>T	p.Arg425Cys	damaging	probably damaging	6:147635147	8,15x10-6 (1/120000)	3,388	father	Novel	***
70102	6	VPS13B	1	c.1298T>C	p.Val433Ala	damaging	benign	8:100146951	2,8x10-5 (1/35000)	4,434	father	Novel	***
70102	6	NRXN3	1	c.2012G>A	p.Arg671His	damaging	probably damaging	14:79434678	3,19x10-4 (1/3100)	6,424	mother	Novel	*
70102	6	TSC2	1	c.4527_4529delCTT	p.Phe1510del	/	/	16:2134981	5,12x10-3 (1/195)	/	mother	Novel	f
85256	4	CEP290	3S	c.4237G>C	p.Asp1413His	damaging (0,007)	benign (0,098)	12:88480233	2x10-3 (1/500)	4,019	mother	Novel	f
85256	4	PCDH9	3	c.2714G>C	p.Ser905Thr	tolerated (0,065)	possibly damaging (0,873)	13:67799859	not present	4,105	mother	Novel	***
85256	4	NRXN3	1	c.1993C>T	p.Arg665Trp	damaging (0,028)	probably damaging (0,994)	14:79434659	not present	0,871	mother	Novel	***
85256	4	DMD	S	c.2391T>G	p.Asn797Lys	tolerated (0,832)	benign (0,203)	X:32509625	6x10-3 (1/150)	0,305	mother	Novel	f
LDM763	2	SMG6	3	c.954_956delGAG	p.Arg319del	neutral (-1,26) (Provean)	/	17:2203090	3,9x10-6 (1/250000)	/	father	Novel	***
LDM763	2	HCFC1	S	c.4442C>T	p.Thr1481Met	damaging	possibly damaging (0,923)	X:153219113	1,1x10-3 (1/900)	2,601	mother	Novel	-
A17/97	3	ASH1L	1	c.8697T>A	p.Ser2899Arg	damaging (0,004)	benign (0)	1:155307986	not present	0,246	father	Novel	***
A17/97	3	MBD5	1	c.2030G>A	p.Ser677Asn	tolerated	benign (0)	2:149227542	2,23x10-3 (1/450)	2,434	father	Novel	f
A17/97	3	ASTN2	2	c.2731C>G	p.Gln911Glu	damaging (0,004)	possibly damaging (0,901)	9:119413995	5,6x10-5 (1/17000)	5,504	father	Novel	***
79149	3	TRIM33	3	c.2801T>C	p.Ile934Thr	damaging (0,01)	benign (0,0939)	1:114945473	2,1x10-5 (1/47000)	4,385	father	Novel	***
79149	3	DLGAP2	3	c.1751C>T	p.Thr584Met	tolerated (0,125)	benign (0,057)	8:1616675	5,8x10-3 (1/172)	1,839	mother	Novel	f
79149	3	CACNA1C	1	c.6062G>A	p.Arg2021Gln	tolerated (0,545)	/	12:2797746	3,1x10-3 (1/300)	3,796	father	Novel	f
D17/73	2	ANK2	1	c.1378A>G	p.Thr460Ala	tolerated	benign (0,017)	4:114179559	3,9x10-6 (1/250000)	2,288	father	Novel	***
D17/73	2	PCDH19	1	c.3280C>G	p.Leu1094Val	tolerated (0,608)	benign (0)	X:99551442	1,4x10-3 (1/700)	0,412	mother	Novel	-
E17/77	3	NSD1	1	c.7025C>T	p.Ser2342Leu	tolerated (0,080)	possibly damaging (0,651)	5:176721394	7,79x10-5 (1/12800)	1,469	father	Novel	***
E17/77	3	DST	3	c.14210A>C	p.His4727Pro	damaging	/	6:56346951	not present	5,246	mother	Novel	***
E17/77	3	CREBBP	1	c.3469G>A	p.Val1157Ile	tolerated (0,574)	benign (0,002)	16:3807950	1,42x10-5 (1/70400)	4,513	mother	Novel	***
F17/16	2	KCTD13	3	c.88G>C	p.Ala30Pro	tolerated (0,142)	benign (0,002)	16:29937267	not present	1,539	father	Novel	***
F17/16	2	ANKRD11	1	c.5573C>T	p.Pro1858Leu	damaging (0,001)	possibly damaging (0,926)	16:89347377	not present	2,387	father	Novel	***
H16/64	6	IMMP2L	3	c.343C>T	p.Arg115Cys	damaging (0,011)	possibly damaging (0,830)	7:110526714	3,97x10-4 (1/2500)	3,955	mother	Novel	*
H16/64	6	CADPS2	3	c.1706T>C	p.Ile569Thr	tolerated	benign (0,002)	7:122130281	3,3x10-4 (1/3000)	2,442	mother	Novel	*
H16/64	6	CSMD1	3	c.3689G>A	p.Arg1230His	tolerated	possibly damaging (0,872)	8:3165968	9,29 x10-5 (1/10700)	2,052	mother	Novel	***
H16/64	6	CHD8	1	c.5282A>G	p.Lys1761Arg	tolerated (0,400)	benign (0.044)	14:21863179	not present	3,249	father	Novel	***
H16/64	6	CHD8	1	c.1843C>T	p.Pro615Ser	tolerated (0,070)	benign (0.012)	14:21883940	1,77x10-3 (1/560)	4,831	father	Novel	-
H16/64	6	SLC16A2	no rating	c.949C>T	p.Arg317Cys	damaging (0)	probably damaging (0,999)	X:73744567	4,44x10-4 (1/2200)	2,392	mother	Novel	*
D17/50	4	ASTN2	2	c.769T>A	p.Ser257Thr	damaging (0)	possibly damaging (0,890)	9:119976883	2,05x10-3 (1/480)	4,962	father	Lionel et al., 2014	f
D17/50	4	CHD8	1	c.5782C>T	p.Arg1928Trp	damaging (0,002)	probably damaging (0,999)	14:21862172	1,61x10-5 (1/62000)	2,748	father	Novel	***
D17/50	4	NRXN3	1	c.3156G>T	p.Gln1052His	damaging (0,014)	benign (0,252)	14:80328277	7,85x10-5 (1/12700)	6,289	mother	Novel	***
D17/50	4	ANKRD11	1	c.6200G>A	p.Ser2067Asn	tolerated (0,315)	benign (0,001)	16:89346750	3,19x10-5 (1/31000)	0,339	mother	Novel	***
E17/49	4	SCN1A	1	c.5783G>T	p.Arg1928Leu	tolerated (0,053)	benign (0,353)	2:166848002	not present	4,742	mother	Novel	***
E17/49	4	DST	3	c.16103A>G	p.Lys5368Arg	tolerated (0.123)	benign (0,012)	6:56323828	3,29x10-4 (1/3000)	1,432	father	Novel	*
E17/49	4	ANKRD11	1	c.5509C>T	p.Pro1837Ser	tolerated (0,380)	benign (0,002)	16:89347441	4,11x10-3 (1/240)	-0,341	father	Novel	f
E17/49	4	ANKRD11	1	c.5338G>A	p.Ala1780Thr	tolerated (1)	benign (0)	16:89347612	4,86x10-3 (1/200)	1,302	mother	Novel	f
LDM1089	3	SCN1A	1	c.5782C>G	p.Arg1928Gly	tolerated	benign	2:166848003	1,3x10-3 (1/769)	1,536	father	Novel	-

LDM1089	3	CADPS2	3	c.1889T>C	p.Met630Thr	damaging	benign	7:122114544	6,26x10-4 (1/1600)	5,163	mother	Bonora et al. 2014	*
LDM1089	3	PCDH9	3	c.5A>G	p.Asp2Gly	damaging	possibly damaging	13:67802568	6,37x10-5 (1/15700)	4,823	mother	Novel	***
D17/27	8	MBD5	1	c.2030G>A	p.Ser677Asn	tolerated	benign (0)	2:149227542	2,23x10-3 (1/450)	2,434	father	Novel	f
D17/27	8	FAT1	3	c.2563G>A	p.Gly855Arg	damaging (0,034)	probably damaging (1)	4:187628419	2,11x10-3 (1/470)	5,809	mother	Novel	f
D17/27	8	FAT1	3	c.56G>C	p.Gly19Ala	tolerated	benign (0,349)	4:187630926	3,68x10-4 (1/2700)	3,816	father	Novel	*
D17/27	8	SLIT3	no rating	c.509G>A	p.Ser170Asn	tolerated	benign (0,368)	5:168271637	7,78x10-5 (1/12800)	3,158	father	Novel	***
D17/27	8	NSD1	1	c.7500G>C	p.Met2500Ile	tolerated	benign (0)	5:176721869	5,68x10-5 (1/17600)	0,052	mother	Novel	***
D17/27	8	IMMP2L	3	c.335A>G	p.Lys112Arg	tolerated (0,549)	benign (0,002)	7:110526722	1,28x10-3 (1/350)	0,755	father	Novel	f
D17/27	8	CSMD1	3	c.2153C>T	p.Ser718Leu	damaging	probably damaging (0,964)	8:3263662	2,82x10-5 (1/35400)	6,068	father	Novel	***
D17/27	8	NRXN3	1	c.3124C>G	p.Gln1042Glu	tolerated (0.357)	benign (0,203)	14:80328245	not present	6,064	mother	Novel	***
F14/24	3	RELN	1	c.5923G>A	p.Gly1975Ser	damaging	possibly damaging (0,887)	7:103194153	9,41x10-4 (1/1060)	5,951	not maternal	Novel	*
F14/24	3	CHD8	1	c.2362G>A	p.Val788Met	tolerated (0,100)	benign (0,005)	14:21878012	9,28x10-4 (1/1077)	2,295	mother	Novel	*
F14/24	3	DMD	S	c.1997C>T	p.Ser666Leu	damaging	probably damaging (0,999)	X:32563447	1,46x10-3 (1/680)	4,954	not maternal	Novel	-
D17/19	5	NRXN1	1	c.2242C>A	p.Leu748Ile	tolerated (0.279)	possibly damaging (0,576)	2:50765412	3,98x10-3 (1/250)	3,101	father	Kim HG , et al. (2008) - Onay H , et al. (2017)- Reale et al. (2021)	f
D17/19	5	SCN2A	1	c.2549G>A	p.Arg850Gln	damaging (0)	probably damaging (1)	2:166198966	not present	6,076	mother	Novel	***
D17/19	5	CSMD1	3	c.119A>G	p.Asn40Ser	tolerated	benign (0,347)	8:4495047	6,4x10-5 (1/15600)	3,033	mother	Novel	***
D17/19	5	VPS13B	1	c.1639A>G	p.Thr547Ala	tolerated	benign (0)	8:100148968	2,26x10-4 (1/4400)	1,083	father	Novel	*
D17/19	5	CACNA1A	1S	c.1242G>T	p.Arg414Ser	tolerated	benign (0,112)	19:13443696	4x10-6 (1/250000)	-0,418	mother	Novel	***
D17/35	4	ANK2	1	c.3529C>T	p.Pro1177Ser	tolerated	benign (0,402)	4:114257151	not present	3,153	mother	Novel	***
D17/35	4	LAMC3	no rating	c.1217C>T	p.Thr406Met	damaging	possibly damaging (0,907)	9:133914569	1,39x10-4 (1/7200)	1,588	father	Novel	**
D17/35	4	BDNF	no rating	c.251C>T	p.Thr84Ile	damaging (0)	probably damaging (1)	11:27680107	1,26x10-3 (1/700)	4,508	father	Novel	-
D17/35	4	ANKRD11	1	c.5509C>T	p.Pro1837Ser	tolerated (0,380)	benign (0,002)	16:89347441	4,11x10-3 (1/240)	-0,341	father	Novel	f
70555	7	CSMD1	3	c.8935G>A	p.Gly2979Ser	damaging (0,005)	probably damaging (1)	8:2824257	5,45x10-3 (1/183)	6,062	mother	Novel	f
70555	7	VPS13B	1	c.3811A>T	p.Thr1271Ser	tolerated	probably damaging (0,997)	8:100493971	7,22x10-4 (1/1385)	4,147	mother	Novel	*
70555	7	VPS13B	1	c.7787C>T	p.Ser2596Phe	damaging (0,006)	possibly damaging (0,877)	8:100791192	7,27x10-4 (1/1375)	3,443	mother	Novel	*
70555	7	KCNMA1	3	c.3150G>A	p.Thr1050=	tolerated (1)	/	10:78651475	2,52x10-4 (1/3960)	-0,068	father	Novel	*
70555	7	CEP290	3S	c.6401T>C	p.Ile2134Thr	damaging (0,004)	probably damaging (0,999)	12:88454728	6,99x10-3 (1/140)	4,649	father	Novel	f
70555	7	MED13L	1	c.1877C>T	p.Pro626Leu	tolerated (0,306)	benign (0)	12:116446341	4,5x10-4 (1/2200)	0,702	father	Novel	*
70555	7	CACNA1H	2	c.5113G>A	p.Ala1705Thr	tolerated (0,060)	probably damaging (0,989)	16:1265315	5,51x10-3 (1/180)	5,251	mother	Novel	f
73664	2	VPS13B	1	c.7753G>A	p.Glu2585Lys	tolerated (0,386)	benign (0,020)	8:100791158	3,15x10-3 (1/300)	1,788	father	Novel	f
73664	2	CACNA1H	2	c.6934A>G	p.Met2312Val	tolerated (1)	benign (0)	16:1270866	1,79x10-3 (1/560)	-0,082	mother	Novel	-
80147	4	RELN	1	c.3477C>A	p.Asn1159Lys	tolerated	probably damaging (0,993)	7:103236965	1,56x10-3 (1/178)	2,229	father	Bonora E , et al. 2003	f
80147	4	ST7	3	c.1700G>T	p.Arg567Leu	tolerated (0,054)	benign (0)	7:116862976	6,42x10-3 (1/155)	0,048	father	Novel	f
80147	4	WNT2	no rating	c.791C>T	p.Thr264Met	tolerated (0,165)	probably damaging (1)	7:116937728	3,53x10-5 (1/28300)	4,017	father	Novel	***
80147	4	VPS13B	1	c.1248G>T	p.Gln416His	damaging	probably damaging (0,998)	8:100146901	1,18x10-3 (1/850)	1,343	mother	Novel	-