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THE DIAGNOSTIC YIELD AND GENETIC CONTRIBUTION OF AUTISM SPECTRUM DISORDER CASES IN QATAR POPULATION

BY

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ABSTRACT

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Autism spectrum disorder (ASD) is a group of clinically and genetically diverse neurodevelopmental conditions. The genetics of ASD has been extensively studied, but the literature on ASD in Qatar is limited. Our study aims to gain new insight into the genetic basis of ASD in Qatar and the diagnostic yield of different genetic tests to improve local and international testing guidelines for ASD. We conducted a retrospective chart review of 301 pediatric cases of clinically confirmed ASD referred to the Medical Genetics department at Hamad Medical Corporation in Qatar between January 2017 and December 2019. This study revealed a male to female ratio of 3.6:1. The clinical presentations of individuals with ASD were classified into five groups: high-functioning ASD, non-verbal ASD, ASD with attention deficit hyperactivity disorder (ADHD), complex ASD, and ASD with developmental delay/intellectual disability (DD/ID). A total of 289 (96%) cases underwent chromosomal microarray analysis (CMA) and 276 (91.7%) cases had Fragile X syndrome (FXS) testing, both as first-tier tests, while only 137 (45.5%) cases had whole exome sequencing (WES) as a second-tier test. Overall, a genetic diagnosis was established in 16 (5.3%) patients with ASD: nine were diagnosed by WES (9/137; diagnostic yield = 6.6%), seven by CMA (7/289; diagnostic yield = 2.4%), and none by FXS testing (0/276; diagnostic yield = 0%). Considering nationality, Qatari patients more commonly underwent WES (p=<0.001), while non-Qatari patients more frequently had CMA (p=0.001), which is primarily explained by the inability of non-Qataris to afford WES. The ASD with DD/ID group had more positive results (p=0.003) by WES, and a higher overall diagnostic yield (p=0.0001)

compared to other ASD groups. Our data provides evidence of a higher diagnostic yield for WES overall and specifically in cases of ASD with DD/ID compared to CMA, which supports the implementation of WES as a first-tier genetic test in such cases. Our data supports a modest role for CMA in the genetic diagnosis of ASD, thus it could still be offered, especially if WES is negative or not accessible to patients. Our data reveals no clinical utility for FXS testing in the absence of a specific clinical suspicion or family history of FXS. Finally, our findings highlight the diversity of the genetic architecture of ASD even in a highly consanguineous population.

Keywords: *ASD, Diagnostic Yield, Fragile X Syndrome, Chromosomal Microarray, Whole Exome Sequencing.*

DEDICATION

This project is proudly dedicated to my father "Waleed", my mother "Basmah" and my brothers "Khaled" and "Moaz"

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Chapter 1: Introduction

Autism spectrum disorder (ASD) is a group of complex neurodevelopmental conditions characterized by the early onset of issues in social communication and interaction that can present with diverse phenotypes. Abnormal responses, language difficulties, mood changes, and repetitive behaviors are the most common signs of ASD [1]. Reflecting the heterogeneity of ASD, the term "autism" is used to describe both a broader presentation as well as a specific diagnosis following its consideration as a subgroup within the general diagnostic category of 'pervasive developmental disorders (PDDs). PDDs are a group of disorders introduced in the Diagnostic and Statistical Manual of Mental Disorders, third edition (DSM-III) in 1980 to convey the idea of a broader spectrum of social communication deficits. Owing to a lack of clear borders between the PDDs and difficulties in reliably distinguishing them, the most recent diagnostic system DSM-V, uses the umbrella term 'ASD' and differentiates individuals using additional clinical specifiers and modifiers [2]. In addition to the DSM-V criteria, the Autism Diagnostic Observation Schedule (ADOS) is also used as a semi-structured standardized diagnostic test for ASD [3]. In the 2010 Global Burden of Disease study, an estimated 52 million people had autism globally, equating to a prevalence of 1 in 132 individuals (1%) [4].

Epidemiological and community-based studies have suggested that ASD is more common in males than in females, with an estimated male to female ratio of 3:1 [5]. However, little is known about the epidemiology of ASD in Arab countries. In Qatar, a study carried out in 2019 estimated a prevalence of 1.14% for ASD among 6 to 11-year-old children [6]. Although ASD was initially assumed to be of environmental origin, an improved understanding acknowledges a particularly large genetic contribution with an estimated heritability of 40% to 90% [7]. More than 100

genes and genomic loci/regions such as 7q, 1p, 3q, 16p, and 15q have been found to be associated with ASD [8]. The advancement of genomic techniques such as Chromosomal microarray (CMA) have shown that several genetic structural variations such as copy number variants (CNVs), may contribute to ASD [9]. CMA is currently recommended as the first genetic testing tier for ASD individuals, and the diagnostic yield ranged from 7.0% to 11% [10, 11]. Whole-exome sequencing (WES), on the other hand, is used to highlight mutations in the etiology of ASD, its diagnostic yield is ranging from 16.7% to 28.6% depending on the clinical presentation of each case [12-14]. WES has a high diagnostic yield in neurocognitive disorders in Qatar and it is better suited for populations with high rates of consanguinity [15] however no clear diagnostic yield is established for ASD yet. Although our knowledge of the genetic basis of ASD has been improved, genotype-phenotype correlation is still challenging due to the phenotypic and genotypic heterogeneity of the disease [16].

This research project aims to contribute to filling the gaps by bringing more insights into the genetic basis and diagnostic yield of different genetic tests and improving the use of local and international guidelines specific to ASD. The primary objectives of this study are:

1- To investigate the genetic makeup of ASD children in Qatar.
2- To evaluate the diagnostic yield of different genetic testing tools in ASD patients in Qatar.

The secondary objective is

3- To explore the genotype-phenotype correlation and impact of consanguinity in ASD patients in Qatar.

Chapter 2: Literature Review

2.1 Background and History

Autism spectrum disorder (ASD) is a group of heterogenous neurodevelopmental conditions that are usually characterized by early-onset social communication issues and can be presented in wide variety of combinations. The main characteristics of ASD are grouped into two main categories: social interaction, and communication impairment, and repetitive actions or interests [1, 17]. The psychiatrist Leo Kanner was the first to use the term autism in 1943 to describe a separate syndrome which he identified in 11 children in his clinic [18]. Although these children were sharing signs of schizophrenia however three main aspects led Kanner to describe them as a separate syndrome which were family history, clear signs of the disorder before the age of 3, and lack of hallucinations. Although Kanner differentiated Autism from Schizophrenia however he described the disorder as the earliest form of schizophrenia. Three characteristics were found by Kanner to describe the disease which were social isolation, language impairments, and insistence on sameness. These characteristics were still used for the next 50 years by the American Psychiatric Association (APA) as defining characteristics of autism [19]. The cause of Autism was described by Kanner as the theory called “refrigerator mother” which described the lack of mother care and emotional warmth to her child is the main reason for developing Autism [18]. However later studies proved a more complicated causation of the diseases. Similarly, Hans Asperger a pediatrician in 1944, published a case series paper of children with similar presentation described as social impairment and stereotyped behaviors [20].

In the last hundreds of years, the definition of autism and diagnostic criteria has changed several times, but the observations of Kenner and Asperger played an

important role in shaping the current definition of autism. Initially, in 1952 the first edition of the Diagnostic and Statistical Manual of Mental Disorder (*DSM-I*) was released by APA to help physicians and physiatrist in evaluating and diagnosing different mental health conditions [21]. *DSM-1* is used to describe autism as a form of childhood schizophrenia. In 1968, *DSM-II* still associated the occurrence of autism with the presentation of childhood schizophrenia, however, the new addition supported that autism might result in mental retardation. Autism was not officially separated from schizophrenia until 1980 when it was labeled as infantile autism with six diagnostic criteria which were required in the *DSM-III* [22]. Later in 1987, the *DSM-IIIR* changed the title of the diagnosis to "Autistic Disorder" and described autism as a "pervasive lack of responsiveness to other people". In 1994, DSM-IV was published, and five main subgroups were introduced under the term autistic disorder through the expansion of the diagnostic criteria which increased to 13. The autism disorder subgroups were divided to Asperger's Disorder, Pervasive Developmental Disorder (PDD) NOS (not otherwise specified), Rett's Disorder, and Childhood Disintegrative Disorder. Asperger autism was understood and described as high functioning autism where patients have specific technical or scientific skills but lack social communication [23]. PDD-NOS was used to group children who present with significant symptoms of one of the three core areas of autism (social communication, language delay and repetitive behaviors), but who do not meet the full criteria of DSM-IV for Autism of other PDD. [24]. Rett's disorder (or Rett's syndrome) is a neurodevelopmental disorder characterized by psychomotor regression, poor communication skills, repetitive behaviors, and uncontrolled movements [25]. Childhood Disintegrative Disorder is used to describe children who develop normally then start to lose their skills at the age of 3-4 years [26]. In 2013, the DSM-V was

published which again changed the definition of autism by collecting the subcategories into one umbrella and used the term Autism Spectrum Disorder “ASD”, moreover differentiating individuals using additional clinical specifiers and modifiers [2].

In DSM-V Rett syndrome was no longer under ASD and is considered as a separated neurological disorder [27] while all other subcategories fall under the umbrella of autism. According to the APA 2013, ASD is now defined when the patient has two categories of the three cores: impaired social communication and/or restricted interaction and/or repetitive behaviors. Although DSM-V is considered the gold standard method in diagnosing ASD, Autism Diagnostic Observation Schedule (ADOS) is used as a semi-structured standardized diagnostic tool for ASD [3].

2.2 Prevalence of ASD

Recent studies have reported that 1 in 132 children are diagnosed with ASD [4] with an estimated male to female ratio of 3:1[5]. Wide variability in prevalence rates was found worldwide due to factors including methodological differences, case findings, diagnostic procedures, sample size, and age groups. In Canada the prevalence of ASD was reported to be 12.2/1000 in 2014-15 for the age group from 1-17 years old [28] while in the United states of America (USA) a study for 11 states in 2016 showed a prevalence of 18.5/1000 for children aged 8 years old [29]. The prevalence in Europe varies from 6/1000 in Germany, reported in 2012 for children ranging from 6 to 11 years [30], to 4.3-7.8/1000 in France as reported in 2015-2016 and depending on the geographical area for children at the age of 10 years old [31], while in Iceland the highest prevalence rate of 31.3/1000 was reported in 2015 for children aged 8 years old [31].

The prevalence of ASD is also varying in the Middle East. In 2016, Mohammadi, *et al* reported prevalence rates of 1.1/1000 for ASD children ranged from 6-9 years old in Iran [32]. In Oman, the prevalence rate was reported to be 2/1000 for ASD children ranged from 5-9 years old in 2011-2018 [33], in Lebanon, a study was conducted in 2014 reported a prevalence rate of 14.3/1000 for children ranging from 1.3-4 years old [34]. In Bahrain, a study in 2013 showed a prevalence rate of 4.3/10,000 for the age group between 2-27 years old [35]. Moreover, in the United Arab Emirates the prevalence was estimated to be 29/10,000 in 2007 for children at the age of 3 years old [36] and in Saudi Arabia prevalence of 3.5/10,000 was reported in 2013 for children aged 3 years old [37]. Qatar is a country with a small population (2.8 million), a cross-sectional two-phase survey study carried out in the period between 2015-2018 estimated a prevalence of 11.4/1000 among 6–11-year-old children [6] which is considered as a high value compared to previously cited Middle Eastern countries except for Lebanon (Table 1).

Globally the prevalence of ASD in the Middle East is lower than what has been found in USA or Europe. However, the prevalence of ASD is increasing worldwide due to the advancement and availability of diagnostic tools and the increased awareness in the last few years. Epidemiological, administrative, and community-based studies have suggested that autism is more common in males than in females, with reported ratios ranging from 2:1 to 5:1, with a true estimate of 3:1 ratio [5]. A study suggested that girls with ASD are more commonly misdiagnosed because they usually tend to mask their social deficit through a process described as “camouflaging” [38] which results in diagnostic gender bias [5]. Another proposed hypothesis of gender difference in ASD prevalence suggested that exposure to high

levels of secreted testosterone in early pregnancy result in cognitive hyper-masculinization of the brain which triggers autistic features later in life [39].

Table 1. Prevalence of ASD Worldwide

Country	ASD Prevalence	Age Group	Diagnostic method	Year	Ref
Canada	12.2/1000	1-7 years old	ICD-9/ ICD-10*	2014/2015	[28]
USA (11 states)	18.5/1000	8 years old	DSMV	2016	[29]
Germany	6/1000	6-11 years old	ICD-10*	2012	[30]
France	4.3-7.8/1000	10 years old	ICD-10*	2015/2016	[31]
Iceland	31.3/1000	8 years old	ICD-10*	2015	[31]
Iran	1.1/1000	6-9 years old	DSMV	2016	[32]
Oman	2/1000	5-9 years old	DSMV	2011-2018	[33]
Lebanon	14.3/1000	1.3-4 years old	M-CHAT**	2014	[34]
Bahrain	4.3/10,000	2-27 years old	DSM-IV-TR	2013	[35]
UAE	29/10,000	3 years old	DSM-IV	2007	[36]
Saudi Arabia	3.5/10,000	3 years old	ASSQ***	2013	[37]
Qatar	11.4/1000	6-11 years old		2015-2018	[6]

*Classification of disease 9th or 10th edition
**The Modified Checklist for Autism in Toddlers
***The Autism Spectrum Screening Questionnaire

2.3 Risk Factors

Though ASD was initially assumed to be of environmental origin, an improved understanding of a particularly large genetic contribution, with estimated heritability ranging from 40% to 90% has been demonstrated [7]. Currently, ASD is considered to be a multifactorial disorder caused by genetic, epigenetic, and environmental factors. Table 2 summarizes the most common environmental factors that were linked to ASD.

2.3.1 Environmental risk factors.

2.3.1.1 Parental age

The association between ASD and advanced parental age has been widely studied. It appears that women's risk of giving birth to a child who develops autism increases throughout their reproductive years [40]. However, paternal age confers an

increased risk for autism when mothers are younger than 30 years old. Moreover, delayed childbearing increases the risk of having an affected child by 4.6% [41]. A recent study showed that every 10 years increase in maternal and paternal age increases the risk of having an affected child with ASD by 18% and 21% respectively [42].

2.3.1.2 Pregnancy conditions and related complications

Medications during pregnancy are one of the risk factors of having an affected child with ASD [43]. Several studies have examined the use of serotonin reuptake inhibitors during pregnancy and the increased risk of ASD in the child and results showed that there is a 50% increased risk in those who took the medication during their pregnancy compared to those who did not [44]. Valproate (a medication used to treat bipolar disorder and epilepsy) has also been proven to be risk factor that increase the chance of having an affected child with autism if used during pregnancy [45]. Other medications including antidepressants, anti-asthmatics, and antiepileptics have been proven to cross the placenta and blood-brain barrier and have a supportive animal model to increase the risk of neurological effects in offspring [46].

Smoking and alcohol consumption during pregnancy has been linked as ASD risk factors for a long time, however new studies and meta-analysis have proved that there is no association between alcohol consumption or smoking and the chance of autism occurrence in the future child [42].

Gestational exposure to chemicals has also been proven as environmental risk factors of ASD such as chemicals including pesticides, phthalates, polychlorinated biphenyls (PCBs), solvents, toxic waste sites, air pollutants, and heavy metals [47].

In addition, maternal infection during pregnancy is one of the proven risk factors of ASD and it is explained by the effect of maternal inflammatory responses

during the infection on the development of the fetus brain [48].

A systematic review on the association of Assisted reproductive technologies (ART) and ASD has been published in 2013 and showed contradictory results. Three out of seven studies supported the link between ART and increased risk of ASD while the other four showed no link [49]. Another systematic review conducted in 2017 described the studies that declined the link between ASD and ART as high-quality studies compared to those supporting the association [42].

2.3.1.3 Parental mental and physical health

The family's mental and emotional well-being plays an important role in the health and well-being of their future child. Several studies supported the link between paternal psychiatric history and ASD. One study showed that positive parental history of schizophrenia increases the risk of having an autistic child by three folds. [50]. Maternal physical health and nutritional intake have been also studied and some evidence was found between folate deficiency and the increased risk of ASD [51]. Bleeding is also associated with a very high risk reaching 81% of having an autistic child [52].

2.3.1.4 Vaccination

A suggested association between certain childhood vaccines and autism has been one of the most contentious vaccine safety controversies in recent years. Many parents suspect that vaccines, particularly measles‑mumps‑rubella (MMR) vaccine and thimerosal ‑ containing vaccines (TCVs), can cause autism [53]. Concern about a possible link between vaccines and autism was initially announced by a publication in *The Lancet* in February 1998. However, recent meta-analysis studies have proved that there is no association between these vaccines and ASD [54], furthermore, they also suggested that these vaccines might play a protective role in developing ASD.

Table 2. ASD Environmental Risk Factors

Environmental risk factors with supported evidence
Neonatal hypoxia
Gestational diabetes mellitus
Valproate during pregnancy
Maternal age >40
Paternal age >50
Sibling with ASD
Maternal obesity
Preterm birth
Serotonin reuptake inhibitors during pregnancy
Gestational exposure to chemicals
Maternal infection during pregnancy
Maternal folate deficiency
Psychiatric family history
Environmental risk factors with inconclusive evidence
Assisted reproductive technologies
Environmental risk factors with the hypothesis that are clearly not supported
Smoking during pregnancy
Alcohol consumption during pregnancy
Caesarian section
Prolonged labor
Vaccination

2.3.2 Genetic risk factors

The genetic contribution to ASD was suggested in the 1970s after monozygotic twin studies showed a 60-70% concordance rate [55] compared to 30% in dizygotic twins [56]. Later studies on heritability proved that the siblings of an affected person have an 18% increased risk of developing autism compared to the normal population and the risk increases to 33% if having two affected individuals within the same family [57]. Twin and family studies consistently demonstrate that autism has a particularly large genetic contribution, with estimated heritability ranging from 40% to 90% [7].

More than 100 genes and genomic regions such as 7q, 1p, 3q, 16p, and 15q have now been confidently associated with autism [8]. The advancement of genomic techniques such as whole exome sequencing (WES) methodologies has shown that genetic structural variation contributes significantly to autism such as copy number

variants (CNV) [9]. ASD is considered a feature of many autosomal dominant, autosomal recessive, X-linked monogenic disorders [58].

Known genetic risk factors (i.e., single gene mutation or CNVs) associated with ASD account for 50% of the total cases compared to 20% of *de novo* variants leaving 30% of the cases with undetermined genetic risk [59].

2.3.2.1 Candidate gene studies

Candidate gene studies aim to explore gene variants that could play a role in the onset of the disease. Genes that are crucial for brain development, synapsis formation, and neurotransmission are a potential autism-causing candidates. The diversity of ASD genetic architecture is known, although the number of genes associated with autism is increasing, the contribution of each gene in the ASD population is minor with none of these genes is found in more than 2% of the ASD population [60]. Different inheritance patterns have been reported with autosomal dominant being the most common type followed by autosomal recessive and rarely X linked or *de novo* [50].

The largest class of genetic risks of ASD accounts for around 40% and 60% in simplex families and multiplex families respectively [61, 62]. It is anticipated to be derived from common variants i.e., single nucleotide polymorphisms (SNPs) of an additive effect, nearly all of which have not be recognized (single nucleotide polymorphisms with allele frequency more than 5% in the general population) [5].

It has been studied that genes would have the most effect on the cellular function when they are highly expressed, this principle was used as a supportive theory to discover genes associated with ASD [63]. Variants in genes associated with brain development especially those involved in neuronal proliferative signaling such as Wnt Family Member 2 gene (*WNT-2*) are potentially associated genes in the

disease onset [64]. Similarly, genes associated with establishing neuronal connectivity have also been linked to ASD including Reelin gene (*RELN*) [65]. Moreover, genes involved in neurotransmissions such as Hydroxytryptamine Receptor 2A (*HTR2A*) [66] and serotonin transporter gene *SLC6A4* have also been identified as ASD candidate genes [67]. Other well-known and studied genes include gamma-aminobutyric acid (GABA) A receptor, beta 3 (*GABRB3*); oxytocin receptor (*OXTR*); N-methyl-D-aspartate receptor (*NMDA*; *GRIN2B*); arginine vasopressin receptor 1A (*AVPR1A*); engrailed homeobox 2 (*EN2*); integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61; *ITGB3*); met proto-oncogene (hepatocyte growth factor receptor; *MET*); and contactin-associated protein-like 2 (*CNTCAP2*) genes [68], have been also identified as candidate genes. Another high confidence ASD risk gene that was identified in a recent study is Chromodomain Helicase DNA Binding Protein 8 *(CHD8)* which is considered the highest number gene identified in *de novo* loss of function (LOF) mutations in ASD patients [69].

Though majority of the gene variations have only a minor influence or impact, the association of these common variants is still useful. Much larger sample sizes are still needed to replicate the findings and identify many novel loci.

In contrast, 1- 5 % of ASD is believed to be caused by rare genetic mutations (minor allele frequency (MAF) <5% of general population) which occur only in a single gene of the high-risk autism associated gene category. For instance, synaptic genes such as neuroligins family *NLGN3 and NLGN4* [70], scaffolding proteins family *(SHANK1, SHANK2, and SHANK3)* as well as neurexin family *(NRXN1 and NRXN3)* [71-76] and others such as *CNTNAP2, SLC9A9, BCKDK, AMT, PEX7, SYNE1, VPS13B, PAH, ADNP and POMGNT1* are known high risk autism associated genes [77-81].

Table 3. Rare Inherited Variations in ASD

Genes	Descriptions	References
Neuroligin family *NLGN3* and *NLGN4*	Rare X-linked mutations in ASD males and mental retardation in several families	[70]
CNTNAP2, SLC9A9 and BCKDK AMT, PEX7, SYNE1, VPS13B, PAH, and POMGNT1	Rare recessive mutations in consanguineous families were described in Amish families and Middle Eastern families with ASD and epilepsy	[77-80]
The scaffolding proteins family (*SHANK1, SHANK2, and SHANK3*) as well as neurexin family (*NRXN1 and NRXN3*) and *ADNP*	Rare inherited variants have been documented in ASD. An association of facial dysmorphic features and ASD was described by mutations in *ANDP* gene	[71-76, 81]

2.3.2.2 Copy number variants (CNVs)

Variations of copy number in DNA as a result of deletion, duplication, or insertion of specific segments account for the normal genetic heterogeneity in the general population [82]. However, CNVs can also be linked to a specific disease, their association in mental disorders has been widely studied and it was proven that they play a vital role in the onset of different neurological disorders [10]. With the advancement of technology and the use of CNA, many studies were able to link different pathogenic CNVs to be contributing factor in the onset of autism which accounts for 11% of the cases [10]. Moreover, pathogenic *de novo* CNVs were reported to be the most common compared to inherited CNVs [83]. Although the presence of *de novo* CNVs in an affected individual supports its contribution to the pathogenesis of the disease, however, multiplex family studies showed inconsistency in genotype-phenotype correlation as some of the CNVs were also found in a healthy individual while being missing from another affected one [84]. These studies supported the idea of the heterogeneity of CNVs linked to ASD and suggested that segregation analysis is recommended as many of these CNVs are related to specific families. Nevertheless, many CNVs were identified in many affected individuals from different families and were strongly associated with ASD (Table 4) [15].

Table 4. Common CNVs Reported in Association with ASD

Chromosomal locus	Genomic coordination	ASD proportion	Penetrance	Ref
1q21.1 Deletion	GRCh38/hg38 chr1: 147, 105, 904–147, 922, 392	10%	Reduced	[85]
15q11.2 Deletion	GRCh38: 15: 20,500,000–25,500,000	27%	Reduced	[86]
15q13.3 Deletion	GRCh38/hg38 chr15: 30,500,000–32,500,000	11%	Reduced but high*	[87]
16p11.2 Deletion	GRCh37/hg19 chr16: 29,606,852–30,199,855	24%	Reduced but high*	[88]
16p12.2 Deletion	GRCh37/hg19 chr16: 29,606,852–30,199,855	46%	Reduced but high*	[89]
17q12 Deletion	hg18 chr17:31,893,783-33,277,865;	4.73%	Strong penetrance	[90]

*Most carrier parents have neurophysiological manifestations

Some other recurrent CNVs were also described to be associated with ASD including 15q11-q13 deletion associated with Prader-Willi/Angelman syndromes, 22q13 deletion associated with Phelan-McDermit syndrome, and 22q11.2 deletion associated with Di-George syndrome [91, 92].

The deletion/duplication in critical regions as mentioned above may result in gene disturbance affecting the gene function by either decreased or overexpression. ASD-associated CNV usually affects regions rich in genes responsible for neuronal development [93].

2.3.2.3 Epigenetics

Epigenetics is a term used to describe a wide range of molecular modifications of DNA or histones that result in activating or inhibiting the expression of specific genes without altering the DNA sequence [94]. Therefore, they are described as non-genetic factors that module the already existing genetic risk factors in the person. In other words, it is the study of environmental exposures that will result in gene-environment interaction which will eventually affect the phenotype. In recent years, epigenetics has been strongly linked to the development of the nervous system and

increased interest in exploring epigenetics markers and their link with ASD [46]. As an example, Rett Syndrome and Fragile X syndrome which are very well studied and known risk factors of autism are a result of epigenetic dysregulation [95, 96]. DNA methylation which is considered a type of epigenetics factor that can change with different exposures to environmental factors was proven to be found in the brains of individuals diagnosed with autism [97]. Furthermore, epigenetics has been found to influence autoimmune responses which also be considered as a risk factor of autism [98].

2.3.2.4 The dilemma of genotype/phenotype correlations

One of the most vital concerns that remain unresolved is the understanding of the correlation among genetic variation and phenotype as same mutations may be associated with diverse ASD phenotype.

However, at any rate, distinctive genetic basis can be correlated with three phenotypic clinical presentations with: 1) ASD with syndromic phenotype due to rare, single-gene changes, 2) Severe with particular phenotype due to de-novo variations in the ASD patient or inherited from asymptomatic carriers 3) Broad ASD phenotypes caused by genetic variations in one or many genes, these genes variations are frequent in the over-all population but causing in heterogeneous clinical phenotypes once reaching an evident level throughout complicated gene-gene and gene-environment connections [99].

2.4 ASD in the Arab world

ASD has been reported from different parts of the Arab world, given the high rate of consanguineous marriages (up to 64% of total marriages) [100].

Some of ASD have been characterized for the first time in the Arab countries. For instance, in Saudi Arabia, WES was employed on 19 trios from singleton Saudi

families with ASD. A total of 47 unique rare variants were identified in 17 trios including 38 which are newly discovered. The common mode of inheritance was found to be either autosomal recessive or X-linked. In addition, 15 ASD novel candidate genes, including 5 (*GLT8D1*, *HTATSF1*, *OR6C65*, *ITIH6* and *DDX26B*) have been identified [100].

Another study including two ASD Middle Eastern multiplex families, homozygous mutations in *AMT, PEX7, SYNE1, VPS13B, PAH, and POMGNT1* genes were identified in familial ASD [101].

A recent study from Qatar showed that monogenic autism was found in 3 (1.8%) children with Rett's syndrome, 3 (1.8%) with Fragile X syndrome, and 1 (0.6%) with tuberous sclerosis. The effect of consanguinity as a risk factor was not found to be significant [102].

Only few studies explored the epidemiology of ASD in the Arab Gulf countries, and none have investigated the burden of the disease on the child, family, or society. More research is needed to better identify the burden and risk factors of autism in Gulf countries [103].

In another study, Al Dewik *et al* identified several mutations in ASD patients utilizing WES through clinical practice [104]. These mutations are summarized in Table 5.

Table 5. Genetic Profiling of 13 ASD Patients in Qatar

Pt no	Consanguinity	Family history	Gene	Disease	MOH	Variant	cDNA	Reference
1	Yes	No	*ANK3*	ASD	AD	p.P1489S p.S2366P	c.4465 C>T c.7096 T>C	Novel
2	Yes	No	*PDE6C*	PDE6C -RD	AR	IVS3-1G>T	c.724-1G>T	Novel
3	Yes	Yes	*SLC9A9*	SLC9A9-related disorder	/	p.E312D	c.936 A>T	Novel

Pt no	Consanguinity	Family history	Gene	Disease	MOH	Variant	cDNA	Reference
4	Yes	No	*ASXL3*	ASXL3 -RD	AD	p.A1461DfsX5	c.4382delC	Novel
5	No	No	*MEF2C*	MEF2C-related disorders	AD	p.K91X	c.271 A>T	Novel
6	No	No	*SHANK3*	SHANK3-related disorder	AD	p.L1370RfsX24	c.4109_4110delTG	Novel
7	No	Yes	*GRIN2A*	GRIN2A – related disorders	AD	p.D1293N	c.3877 G>A	Novel
8	No	Yes	CHD2	CHD2 -RD	AD	p.G1651D	c.4952 G>A	Novel
9	No	Yes	*ATRX*	ATRX -RD	X-Linked	p.M2456EfsX41	c.7366_7367delAT	Novel
10	No	No	*MECP2*	Rett Syndrome	X-Linked	p.T158M	c.473 C>T	[105, 106]
11	No	No	*MECP2*	Rett Syndrome	X-Linked	p.R294X	c.880 C>T	[107-110]
12	No	No	*MECP2*	Rett Syndrome	X-Linked	p.P389X	c.1164_1207del44	[111]
13	Yes	Yes	*VPS13B*	Cohen syndrome	AR	p.S3970QfsX22	c.11907dupC	[112, 113]

Pt no= patient number/ MOH= mode of inheritance/ AD= autosomal dominant/ AR= autosomal recessive

2.5 Diagnosis

Autism is typically diagnosed at the age of 3 years old, although the signs might not manifest until later in childhood [114]. The diagnostic assessment of suspected ASD children is a complex process in which it corporate information gathered from the child's parents about his development history and current functioning level as well as observation by an experienced multi-disciplinary team (MDT). Clinical assessment and judgment is the gold standard process of diagnosing children with ASD [115]. To meet DSM-V criteria for ASD, patients are required to meet all three sub-criteria (Table 6) within the social interaction and social communication domain, and two out of four of the sub-criteria within the repetitive and restricted patterns of activity, behaviors, and interest's domain while being assisted by the MDT [116]. The MDT decision is enough to establish the diagnosis of

ASD however, several tools might also be used along with the clinical assessment of the child.

Table 6. DSM-V Diagnostic Criteria for ASD

Domain A. Social communication and social interaction Must have evidence across multiple contexts of all the following three subdomains currently or by history:
1. Deficit in social reciprocity 2. Deficit in Non-verbal communication 3. Deficit in developing, maintaining, and understanding relationships
Domain B. Restricted, repetitive behaviors and interests Must have evidence of two of four of the following subdomains currently or by history:
1. Stereotyped, repetitive behaviors 2. Insistence on sameness 3. Highly restricted, fixed interests 4. Hypersensitivity or hyposensitivity or interest in sensory inputs
Domain C. Symptoms must be present in the early developmental period (but may not become fully manifest until social demands exceed limited capacities or may be masked by learned strategies in later life).
Domain D. Symptoms cause clinically significant impairment in social, occupational, or other important areas of current functioning.
Domain E. These disturbances are not better explained by intellectual disability (intellectual developmental disorder) or global developmental delay. Intellectual disability and autism spectrum disorder frequently co-occur; to make comorbid diagnoses of autism spectrum disorder and intellectual disability, social communication should be below that expected for general developmental level.

*Retrieved from https://www.autismspeaks.org/autism-diagnosis-criteria-dsm-5

The most common ASD assessment tools used is known as autism diagnostic observation schedule (ADOS) which is considered a semi-structured assessment tool that was published in 2000 by the western psychological service (WPS) [117]. The tool is divided into four main modules that are designed to assist the children based on their age and functional group. After ADOS assessment the child will be given a score and the threshold varied between models depending on the age of the child [118]. Autism diagnostic interview, revised (ADI-R) is another tool used in the diagnostic process, unlike ADOS that focuses on the engagement of the child, ADI-R and the developmental history of the child by interviewing people closest to him such as his parents [119]. Together, MDT assessment and the usage of ADOS and ADI-R have

been proven to be the most accurate way of diagnosing ASD [120].

2.6 Clinical heterogeneity of ASD

Variability in ASD clinical presentation is related to the wide variety of observed symptoms, including age of onset and severity of the disease. Comorbidities include morphological (i.e. macrocephaly, microcephaly, dysmorphic features) neurological (i.e. epilepsy, delayed motor development, intellectual disability, and global developmental delay) [121] and psychiatric disorders (i.e. depression and anxiety) are important factors that play a role in the clinical presentation of the patient. Intellectual disability and language impairment are the most common associated comorbidities in ASD patients (70% and 30% respectively) where language impairment varies from difficulties using specific language to complete loss of verbal communication [122].

Some chromosomal microdeletions are known to cause “syndromic ASD” where autism occurs because of other medical conditions (i.e. Di-George syndrome). Beside ASD these patients usually present with dysmorphisms and congenital anomalies (i.e. hypotonia, intellectual disabilities, developmental delay, and other manifestations) and are usually identified clinically before genetic testing where targeted testing for a specific condition might be initiated [91, 92].

ASD can also overlap with some genetic syndromes that include fragile X syndrome, Down syndrome, Rett syndrome, Neurofibromatosis 1, Cohen syndrome, Prader Willi syndrome, phenylketonuria, methyl-CPG-binding protein 2 (MECP2) spectrum disorders, and phosphatase and tensing homolog (PTEN)–related conditions [50]. Results of a systematic review and meta-analysis study that was done in 2015 to show the prevalence of ASD in different genetic disorders are demonstrated in Table 7.

Table 7. Monogenic Syndromes Associated with ASD

Syndrome	Prevalence of ASD	Mutated gene
Rett Syndrome	61%	*MECP2*
Tuberous sclerosis complex	36%	*TSC1/2*
Angelman syndrome	34%	*UBE3A (15q11-q13)*
Down syndrome	34%	*Trisomy 21*
Fragile X syndrome	22%	*FMR1*
Neurofibromatosis (Type 1)	18%	*NF1*
Noonan syndrome	15%	*PTPN11*

Fragile X syndrome (FXS) is an X-linked known genetic syndrome caused by the alteration of the Fragile X Mental Retardation 1 *(FMR1)* gene and it is considered as the most common inherited mental retardation cause [123]. The alteration in the *FMR1* gene usually results from expansion in the CGG repeats (>200 repeats for full mutation) located in the 5' untranslated region of the *FMR1* gene, by which it affects the function of the gene which results in reduced synaptic strength [124]. Boys are usually more affected by FXS as they only have one copy of the X chromosome while females could be a carrier and more mildly affected than males. The affected individuals might show a wide variety of symptoms ranging from normal IQ with mild learning difficulties to severe mental retardation [125]. Approximately 30% of males with FXS are also diagnosed with ASD, while those who are not fully diagnosed with ASD have at least one or more autistic features [126]. Although premutation carriers (55-200 repeats) and ASD has been known as occasional occurrence, a study showed that 14% of premutation males and 5% of premutation females had developed ASD [127]. Premutation alleles carriers are also at risk of developing fragile X associated primary ovarian insufficiency (FXPOI) and fragile X-associated termer ataxia syndrome (FXTAS) [128, 129]. Among all ASD cases, approximately 1-6% of them are diagnosed with FXS [130].

This heterogeneity supports the idea of pharmacologically and behaviorally

treating the associated symptoms and co-occurring conditions rather than treating ASD as a single medical condition [131].

2.7 Molecular diagnosis of ASD

The increased prevalence of ASD has resulted in a massive increased number of clinically diagnosed cases that are usually referred to clinical genetics or genetic counselor for a further case evaluation. Although the genetic contribution in ASD is not fully understood, however, 20-25% of the cases can be genetically diagnosed [132]. The main role of the medical geneticist is to identify the disease etiology and assess the patient clinically (i.e. presence of specific dysmorphic features) to identify any suspected underlying genetic syndrome while the role of geneticist along with the genetic counselor is to provide genetic counseling about the case, facilitate test options, deliver accurate risk assessment to the patients and their families, and improve case management.

In 2013 the American College of Medical Genetics (ACMG) has published a clear guideline for genetic evaluation and testing for children with ASD. The guideline state that clinical evaluation should be offered to all patients diagnosed with ASD as it will help identify and differentiate between sporadic and syndromic ASD which will make the test options clearer and more precise according to the clinical presentation of the case [133] (Figure 1).

2.7.1 Chromosomal microarray

CMA is a whole-genome screening technique also known as comparative genomic hybridization (CGH) used to identify copy number variations in the cytogenetic banding by identifying chromosomal imbalances as well as submicroscopic deletions and duplications referred to as CNVs [134]. The increased sensitivity of CMA compared to other traditional cytogenetic techniques such as

karyotyping (KT) has resulted in considering it as a first-tier testing for multiple conditions (i.e. intellectual disability, developmental delay, multiple congenital anomalies, etc.) [135]. As first-tier genetic testing for children with ASD, the ACMG recommends CMA for all children with ASD which has improved the diagnostic yield to 11% [133].

2.7.2 Fragile X syndrome and single gene sequencing

For all boys who are diagnosed with ASD, it is recommended to test for fragile X syndrome (1-5% diagnostic yield). While it is recommended to test girls only if there is a positive family history [133].

Mutations of methyl-CPG-binding protein 2 (*MECP2)* which were originally linked to Rett Syndrome showed later a broader range of phenotypes including idiopathic ASD in girls (4% diagnostic yield), thus it is recommended to be sequenced in all ASD girls. However, no clear evidence supported the sequencing of *MECP2* in boys unless additional features (other than ASD) of *MECP2* duplication were noted in the male (i.e. drooling, recurrent respiratory infections, hypotonic facies). Phosphatase and tensin homolog (*PTEN)* gene sequencing is recommended for both boys and girls in the case of macrocephaly (> 2.5 SD) as many studies supported the link between pathogenic variants in the *PTEN* gene and ASD. No link between ASD and macrocephaly was identified however all positive *PTEN* cases were found to have macrocephaly [133].

2.7.3 Whole-exome sequencing

When first-tier genetic testing is unable to detect ASD risk-related, WES, which is a technique used to sequence all protein-coding regions within the human genome, can identify genetic basis in up to 20% of the cases [136].

In summary, chromosomal microarray analysis is recommended as the first-

genetic test for individuals diagnosed with ASD while WES is used to highlight *de novo* mutations in the etiology of ASD, its diagnostic yield ranges from 16.7% to 28.6% depending on the clinical presentation of each case [12-14]. In 2019, the American Academy of Pediatrics recommended genetic testing for all children with ASD.

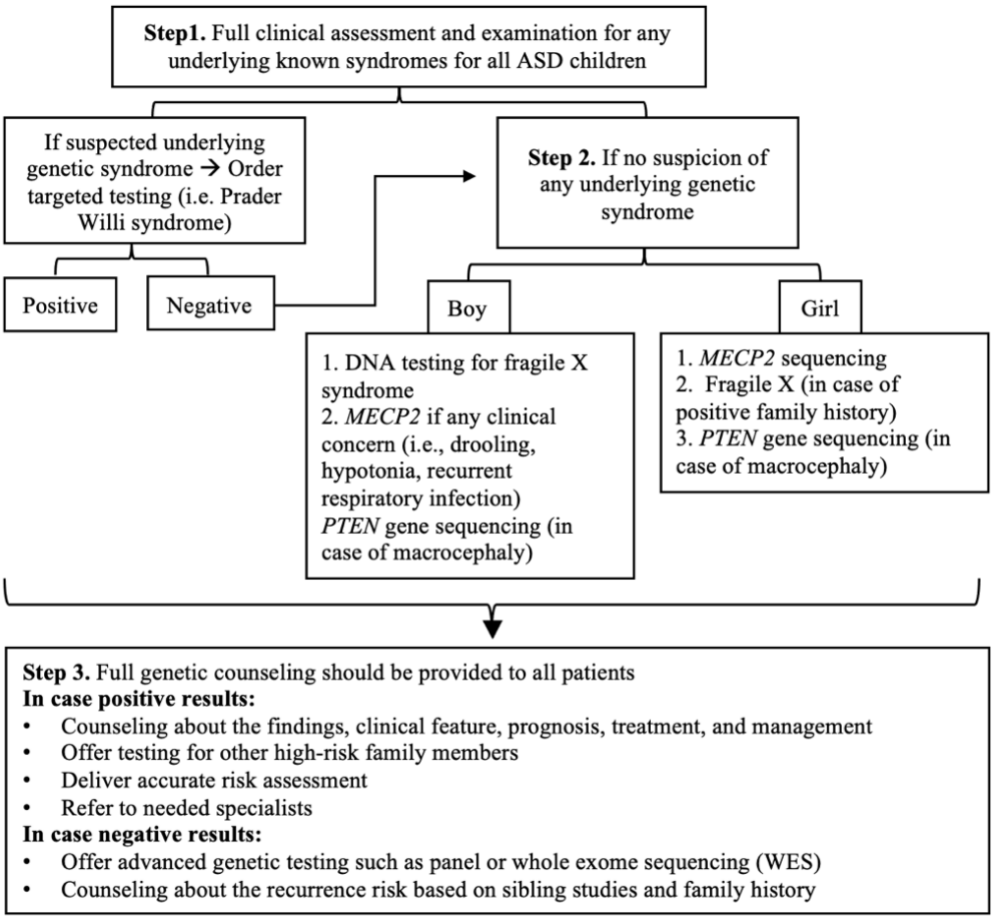

Figure 1: ACMG (2013) recommendation of genetic testing for ASD patients

WES has a high diagnostic yield in neurocognitive disorders in Qatar and is better suited for populations with high rates of consanguinity [15]; however, no clear diagnostic yield is established for ASD patients in Qatar. Although our knowledge of the genetic basis of ASD has improved, genotype-phenotype correlation is still

challenging due to the phenotypic and genotypic heterogeneity of the disease [16].

2.7.4 Metabolic disorders

Although metabolic disorders associated with ASD are relatively very rare and are usually present early in life and detected from birth, the majority of them are associated with symptoms such as seizures, failure to thrive, dystonia, and other symptoms typical of an autistic patient. No studies have linked any diagnostic yield of metabolic disorders and their association with ASD. Thus, testing for the underlying metabolic disorder was left to the decision of the medical geneticist who can identify clear family history and assess the need for any metabolic workup such as when there is multi-systemic involvement (hepatic, renal, cardiac) [133].

2.8 Management, Treatment and Genetic Counseling

Similarly to other neurodevelopmental disorders, no direct cure is available for ASD and no pharmacological treatment for ASD is yet found; however, early interventions and supportive therapies play an important factor in improving child development [137]. Once ASD is clinically diagnosed, clinical interventions include occupational therapy that focuses on improving everyday activities and self-care, behavioral therapy, language and speech therapy, and psychiatric interventions if needed [138]. The goal of management is to maximize functional independence and to promote socialization to improve the quality of life of the affected person and his/her family. Educational intervention such as academic and social support are of the most important strategies used with ASD patients that can address their communication, social, and daily living skills [139].

Globally there are many approaches for improving the life of ASD patients, and many intervention programs were introduced and their efficiency in improving the daily life of autistic patients has been proven. An example is The Early Start

Denver Model (ESDM) [140] that is based on Applied Behavioral Analysis (ABA), directed for ASD patients between the age of 12-48 months. This model was designed not only for health care providers but also includes parents' involvement as a key part of the program [141]. ASD patients may receive medications to treat other associated comorbidities along with ASD such as attention deficit hyperactivity disorder (ADHD), seizures, and psychiatric disorders [142].

Although genetic testing may not always contribute to the treatment or management plan, however it can provide the family with accurate recurrence risk estimates and prognoses. The process of genetic counseling is very important for individuals and their families as the role of the genetic counselor (GC) includes; clinical assessment of the patient, taking a detailed family history, explaining the contribution of genetics in ASD, discussing test options, risks and limitation and disclosing genetic results and prognosis [143]. In the future, genetic testing may not only contribute to the diagnosis of ASD however it can play an important role as a screening tool for those at risk of developing ASD. Moreover, understanding and identifying the genetic basis of the disease will also help in the development of specific genetic-based treatments [144].

Chapter 3: Materials and Methods

3.1 Sample

This study was retrospectively conducted at the Medical Genetics department, Hamad Medical Corporation (HMC) in Qatar. The study has been approved by the Institutional Review Board at HMC (IRB number: MRC-01-21-667) and Qatar University (IRB number: QU-IRB 1609-E/21).

This study included chart review of ASD children referred from Child Development Center (CDC) to the genetics clinic. The list of referred ASD cases was screened by two stages; primarily, the referral list was screened and only cases who were referred from the period of January 2017 to December 2019 were considered for secondary screening by the following criteria:

Inclusion criteria:

- All ages were included
- Patients who were clinically diagnosed with ASD (DSM-V, ADOS or ADI-R)
- Patients who at least completed one genetic testing (FXS/ CMA/ WES).

Exclusion criteria

- Patients who were not seen by the genetics team (no show from the patient side or rejected referral)
- Patients with brain anomalies/malformations
- Patients with neurological disorders (Epilepsy and Seizures)
- Patients with structural congenital defects or dysmorphic features

3.2 Data collection

For patients who met our inclusion and exclusion criteria, their demographics and medical information were abstracted from their charts. All information was recorded whether subjects has completed all their genetic testing or not along with all

results related to FXS testing, CMA and WES. Every patient's data was coded numerically, and no subject identifiers were shared outside the research team at HMC. Data collection sheet was stored as a soft copy within password-locked computers at the HMC PIs office. The research number assigned to each case has no relationship with any information found in the patient medical charts.

After reviewing the medical records of the included patients, they were divided into five different groups based on their ASD presentations and associated phenotypes (Table 8).

Table 8. Classification of ASD phenotypes into Five Main Groups

High functioning ASD	Non-verbal ASD	ASD and Hyperactivity/ ADHD	ASD complex	ASD and DD/ID
Patients with the mildest level of severity of the autism spectrum who can speak and do their daily tasks. The only challenge is social communication	Patients with moderate to severe receptive and expressive speech and/or language delay	Patients with hyperactivity or Attention-Deficit Hyperactivity Disorder	Patients with both speech delay and ADHD, in addition to some other behavioral issues including aggressive behavior	Patients with developmental delay/ intellectual disability, with or without other behavioral issues (speech delay, ADHD, or aggressive behavioral)

3.3 Molecular genetic testing

FXS and CMA testing were performed by the Molecular and Cytogenetic Laboratory, Doha, Qatar. While WES was performed in an abroad laboratory (GeneDx). FXS and CMA testing were either requested by the refereed physician or ordered by the medical geneticist as first-tier genetic testing.

3.3.1 Fragile X syndrome testing

The methodology of FXS testing was performed by polymerase chain reaction (PCR) of the CGG repeats, 5' of *FMR 1* gene followed by fluorescence fragment analysis using the Asuragen Amplidex PCR/CE FMR1 kit (Austin, USA) [145].

Owing to the technical limitations of the test, the number of CGG repeats could be within +/-1 repeats. Results were interpreted by the laboratory according to the ACMG recommendations as following 1) Normal: 5-44, 2) Intermediate (grey zone): 45-54, 3) Premutation: 55-200, and 4) Full mutation: >200 [146].

3.3.2 Chromosomal microarray testing

The genome-wide oligonucleotide array-based comparative genomic hybridization (aCGH) analysis was performed with the use of the Human Genome CGH Microarray kit (designed by Oxford gene technology) [147]. The array contains ~180,000 DNA oligonucleotide probes spaced approximately 30-37 kb apart genome wide. The probe sequences and locations are from the human genome build (hg19). This technique is not able to detect balanced alterations (reciprocal translocation, inversions, Robertsonian translocations and balanced insertions). According to the laboratory reporting approach, some copy number changes may not be reported if they are interpreted as clinically neutral as per the database of Genomic Variants (projects.tcag.ca/variation); however, this information is available upon request. Some genomic imbalances <500 kb may not be reported if there is insufficient published information on gene content. Heterozygous deletions of regions associated with autosomal recessive disorders are not routinely reported.

Findings of CMA were classified by the laboratory based on information gathered from the Database of Genomic Variants (www.projects.tcag.ca/variation) Online Mendelian Inheritance in Man (www.ncbi.nlm.nih.gov/sites/entrez), International Standard Cytogenetic Array Consortium. (http://isca.genetics.emory.edu/iscaBrowser/) and Database of Chromosomal Imbalances and Phenotype in Human using Ensemble Resources (DECIPHER) (https://decipher.sanger.ac.uk.). Results were reported as follows according to internal

reporting approach: 1) Likely pathogenic (when the finding is disease-causing however not enough clinical information is available to correlate with the phenotype), 2) Abnormal (disease-causing findings that correlated with the reported phenotype), 3) Benign (not disease-causing), 4) Likely benign (literature doesn't support the association), and 5) VUS (variant of uncertain significance). Variant reporting follows the International System for Human Cytogenetic Nomenclature (ISCN) guidelines [148].

3.3.3 Whole exome sequencing

After completing the first tier-genetic testing (CMA and FXR), ASD patients and their families were offered WES testing. WES was offered free of charges for national while residents had to cover the price of testing themselves as the test is usually sent to an abroad lab (GeneDx) and not done locally.

Genomic DNA (gDNA) was enriched for the complete coding regions and splice site junctions for 22K genes which accounts approximately 2% of the human whole genome using a proprietary capture system developed by GeneDx for next-generation sequencing with CNV calling (NGS-CNV). The enriched targets were simultaneously sequenced with paired end reads on an Illumina platform [149]. Bi-directional sequence reads were assembled and aligned to reference sequences based on NCBI RefSeq transcripts and human genome build GRCh37/UCSC hg19 [150]. A custom-developed analysis tool "Xome-Analyzer" by GeneDx which consist of, a variant annotation, filtering, and viewing interface for WES data, which includes nucleotide and amino acid annotations, population frequencies (NHLBI Exome Variant Server, 1000 Genomes, and internal databases), in silico prediction tools, amino acid conservation scores, and mutation references [151] was used to report and classify variants. Using this tool, data were filtered and analyzed to identify sequence

variants and most deletions and duplications involving three or more coding exons [152]. Smaller deletions or duplications were not reliably identified. Reported clinically significant variants were confirmed by an appropriate orthogonal method in the proband and, if submitted, in selected relatives as necessary. Sequence and copy number variants were reported according to the Human Genome Variation Society (HGVS) [153] or ISCN guidelines [148], respectively.

Reportable variants include pathogenic variants, likely pathogenic variants, and VUS. Likely benign and benign variants, if present, are not routinely reported. Sequence variants are classified based on the ACMG/AMP guidelines [154]. Known or expected pathogenic variants in the genes recommended by the ACMG SF v2.0 will be reported for the patient in case they opt-in for secondary findings, per the reporting structure recommended by the ACMG [155, 156].

3.4 Assessment of diagnostic yield of the genetic studies

3.4.1 Positive /Negative results

The results of CMA or WES were grouped into 1- Positive and 2-Negative. Results were considered positive if likely pathogenic/ pathogenic variants or VUS were reported while results were considered negative if benign, likely benign and no variants were reported regardless to its diagnostic yield i.e. explaining the phenotypes. This strategy was employed to have an overall picture of findings. As those who recived positive reults are more subjected to have variants that are either disease causing or prone to be recalssifed (i.e. VUS) as disease causing compared to those with negative results. These findings can be used to link the phenotype of the patient with the possibility of reciving posivie results by CMA/WES.

3.4.2 Variant classification

For statistical purposes and to homogenize the results, variants reported from

either CMA or WES were stratified into three groups: 1- Pathogenic variants, 2-Benign variants and 3-Variant of uncertain significance (VUS). The pathogenic variants group includes likely pathogenic, pathogenic variants, and abnormal CNVs, the Benign variants group includes likely benign and benign variants. VUSs were in a seprate group as these include variants that have no established association with reported phenotypes or have strong evidence not being the cause of a disease in the literature according to the joint recommendations of ACMG and Clinical Genome Resource (ClinGen) variant classification and interpretation guidelines [157]. VUSs were classified based on scoring system according to the ACMG gudlines, if the variant failed to fall within the two gategories: “bening” and “pathogenic” this variant was reported as VUS [154].

3.4.3 Solved /Unsolved VUS identified by CMA and WES

Two different approaches were used to label VUS as solved/unsolved depending on the genetic test. For ASD cases with VUS identified by CMA were divided into two groups; unsolved for those variants who were not identified to be inherited (paternally or maternally) nor *de novo* because parental testing was not done. While solved cases were considered “likely benign” for those familial variants who were proved to be inherited from one healthy parent.

ASD cases with VUS by WES were considered solved, if the identified variants are well segregated through the family members i.e. the VUS presents in the index case and other affected family members while absent in the healthy siblings; absent of variants at the parental level for *de novo* variants, in addition to the support of *in-silico* studies reported by the laboratory. The “solved” cases with VUS in our study were also determined through clinical correlation by a group of expert clinical geneticists, genetic counselors, and clinical laboratory scientists. Otherwise, these

cases were labeled “unsolved”.

3.4.4 Diagnostic/Non-diagnostic variants

For the assessment of CMA/WES diagnostic yield, only those who received pathogenic variants were considered, as these variants have strong evidence to be associated with the disease [157, 158]. Since the aim of this study is to identify the diagnostic yield related to ASD and known associated phenotypes, these variants were further classified into diagnostic and non-diagnostic. A variant was considered diagnostic if it was related to either one of the ASD-related clinical phenotypes in the patient such as direct relation to ASD, behavioral problems, or intellectual disability/ developmental delay. Variants were considered non-diagnostic if the associated phenotype of the variant is not related to ASD i.e. secondary findings.

3.5 Statistical analysis

The data is reported as the number of individuals or proportion of subjects within a group. Comparisons of the differences between the groups’ characteristics (age, gender, nationality, family history, etc.) and yield of molecular diagnosis were performed using Fisher exact test with/without post hoc comparisons. *P* value for post hoc test was corrected for 10 simultaneous comparisons using Bonferroni adjustment. A significance level of $\alpha < .05$ was considered for the primary fisher test and $\alpha < .005$ was considered after Bonferroni correction. All statistical analyses were conducted using SPSS Statistics software version 28.0.0.0. Chart figures were conducted using Excel.

Chapter 4: Results

4.1 Referral, inclusion/exclusion, and demographics

The initial screening step started with 1050 cases who were referred to the Medical Genetics department. After excluding 416 cases of those who did not meet the date criteria, the primary screening step revealed a total of 634 ASD cases that had been referred from Child Development Center (CDC) to Medical Genetics department from the period of January 2017 to December 2019. These cases were referred for variety of reasons, including family history of ASD, recent clinical diagnose of ASD, and as suspected ASD case.

The 634 cases were screened for the inclusion/exclusion criteria as mentioned in chapter 3. Of the cases, 591 ASD cases were clinically diagnosed with ASD by DSM-V, ADOS or ADR-I while 43 were excluded as they were not clinically diagnosed. In addition, of the 459 cases seen by the genetics team 114 were excluded for one of the following reasons: the patient had brain anomalies/malformations and/or neurological disorders and/or structural congenital defects/dysmorphic features. Of the 345 cases left, 44 patients did not complete any genetic testing and were excluded. Leaving a total of 301 ASD cases to be studied (Figure 2).

A total of 301 ASD patients were included in the study (Qatari: N=69, 22.9%; Non-Qatari: N= 232, 77.1%). The male to female ratio was 3.6:1 (Female: N = 66, 21.9%; Male: N = 235, 78.1%). Patient's age ranged from 2 to 17 years with a mean age of 5.13 ± 2.58 (Table 9). Parental consanguinity and a positive family history were reported in 30.6% and 30.6% of cases, respectively. The backgrounds of the probands included nationals from Qatar (22.9%), other Arab countries (36.3%) and non-Arabs (40.8%) with total of 77.1% who were non-Qataris from 33 different countries. Egyptians, Filipinos, and Sudanese were the most common non-Qatari

nationalities with frequency of 12.3%, 11% and 11% respectively.

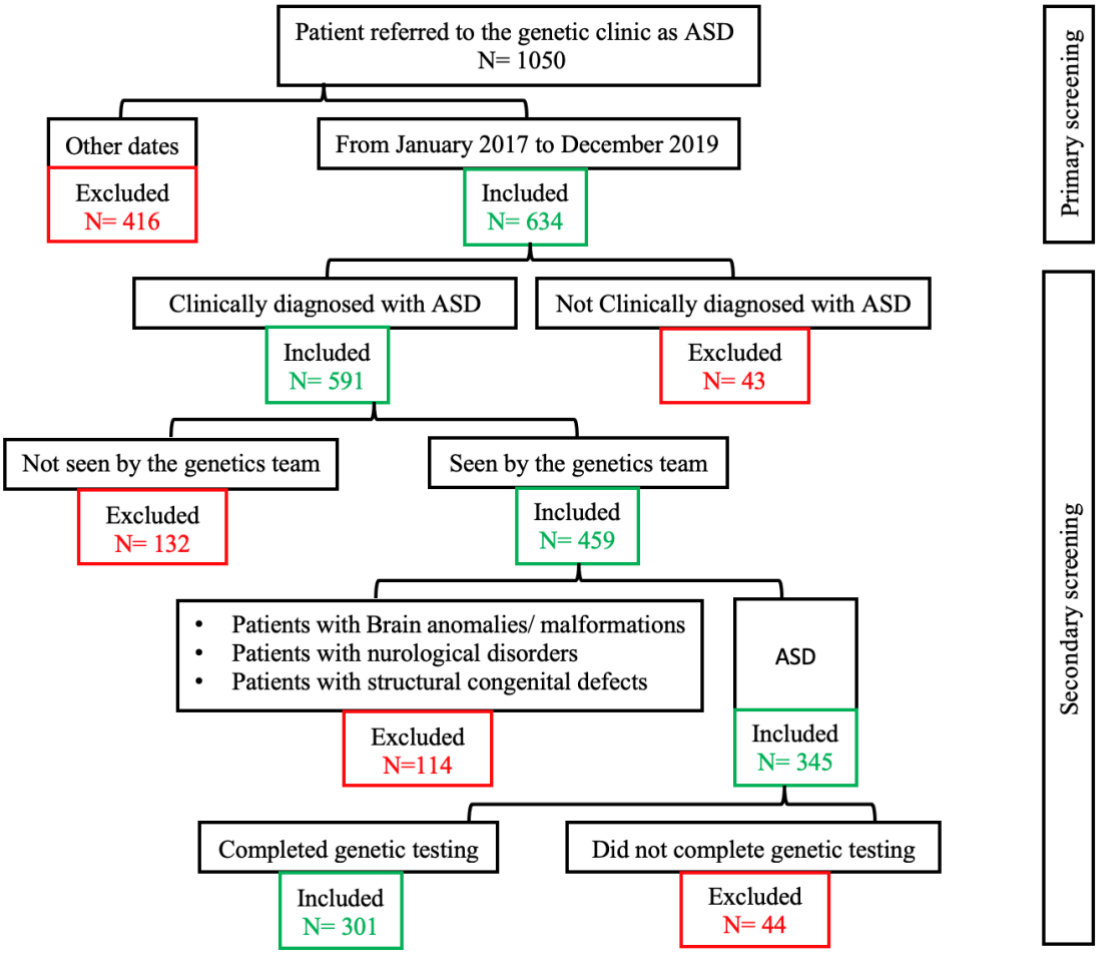

Figure 2. Data collection workflow

N= number of patients

Of the total sample 42/301 patients had co-occurring medical conditions summarized in Figure 3, however none of these patients had a medical condition related to the brain, neurological disorder, or congenital malformation thus they were not excluded and were considered as co-occurring medical conditions that will not affect the diagnostic yield [159]. ASD patients were classified into five groups: 12 (4%) were high functioning ASD group, 107 (35.6%) were non-verbal ASD, 17 (5.6%) were ASD with ADHD, 133 (44.2%) were ASD complex and 32 (10.6%)

were ASD and DD/ID. The demographic data of each group separately are summarized in Table 9. No statistical significance was found for any of the mentioned parameters between the five groups.

Table 9. Demographics of 301 ASD Children According to The Five Groups

		Group						
Factor		**All sample**	**High Functioning ASD**	**Non-Verbal ASD**	**ASD and ADHD**	**ASD Complex**	**ASD and DD/ID**	***p*-Value**
N		301	12	107	17	133	32	
Age (Mean ± SD)		5.13 ± 2.58	5.41 ± 2.99	4.35 ± 1.62	6.12 ± 2.71	5.15 ± 2.40	7.03 ± 4.21	
Gender N (%)								
	Female	66 (21.9%)	2 (16.7%)	30 (28.0%)	1 (5.9%)	27 (20.3%)	6 (18.8%)	0.269
	Male	235 (78.1%)	10 (83.3%)	77 (72.0%)	16 (94.1%)	106 (79.7%)	26 (81.3%)	
Male-Female ratio								
		3.6:1	5:1	2.6:1	16:1	3.9:1	4.3:1	
Nationality N (%)								
	Qatari	69 (22.9%)	4 (33.4%)	18 (16.8 %)	4 (23.5%)	37 (27.8%)	6 (18.7%)	0.252
	Non-Qatari	232 (77.1%)	8 (66.6%)	89 (83.2%)	13 (76.5%)	96 (72.2%)	26 (81.3%)	
Parental Consanguinity N (%)								
	No	209 (69.4%)	9 (75.0%)	74 (69.2%)	11 (64.7%)	95 (71.4%)	20 (62.5%)	0.851
	Yes	92 (30.6%)	3 (25.0%)	33 (30.8%)	6 (35.3%)	38 (28.6%)	12 (37.5%)	
Family History N (%)								
	Negative	209 (69.4%)	6 (50.0%)	77 (72.0%)	11 (64.7%)	91 (68.4%)	24 (75.0%)	0.531
	Positive	92 (30.6%)	6 (50.0%)	30 (28.0%)	6 (35.3%)	42 (31.6%)	8 (25.0%)	
Medical Conditions* N (%)								
	Not present	259 (86.0%)	12 (100.0%)	90 (84.1%)	14 (82.4%)	116 (87.2%)	27 (84.4%)	0.631
	Present	42 (14.0%)	0 (0.0%)	17 (15.9%)	3 (17.6%)	17 (12.8%)	5 (15.6%)	
Testing received N (%)								
	FXS	276 (91.7%)	12 (100%)	97 (90.6%)	15 (88.2%)	126 (94.7%)	26 (81.2%)	
	CMS	289 (96.0%)	10 (83.3%)	105 (98.1%)	16 (94.1%)	129 (96.7%)	29 (90.6%)	
	WES	137 (45.5%)	6 (50%)	38 (35.5%)	7 (41.1%)	62 (46.6%)	24 (75.0%)	

*A total of 26 different medical condition were identified and summarized in figure 3.

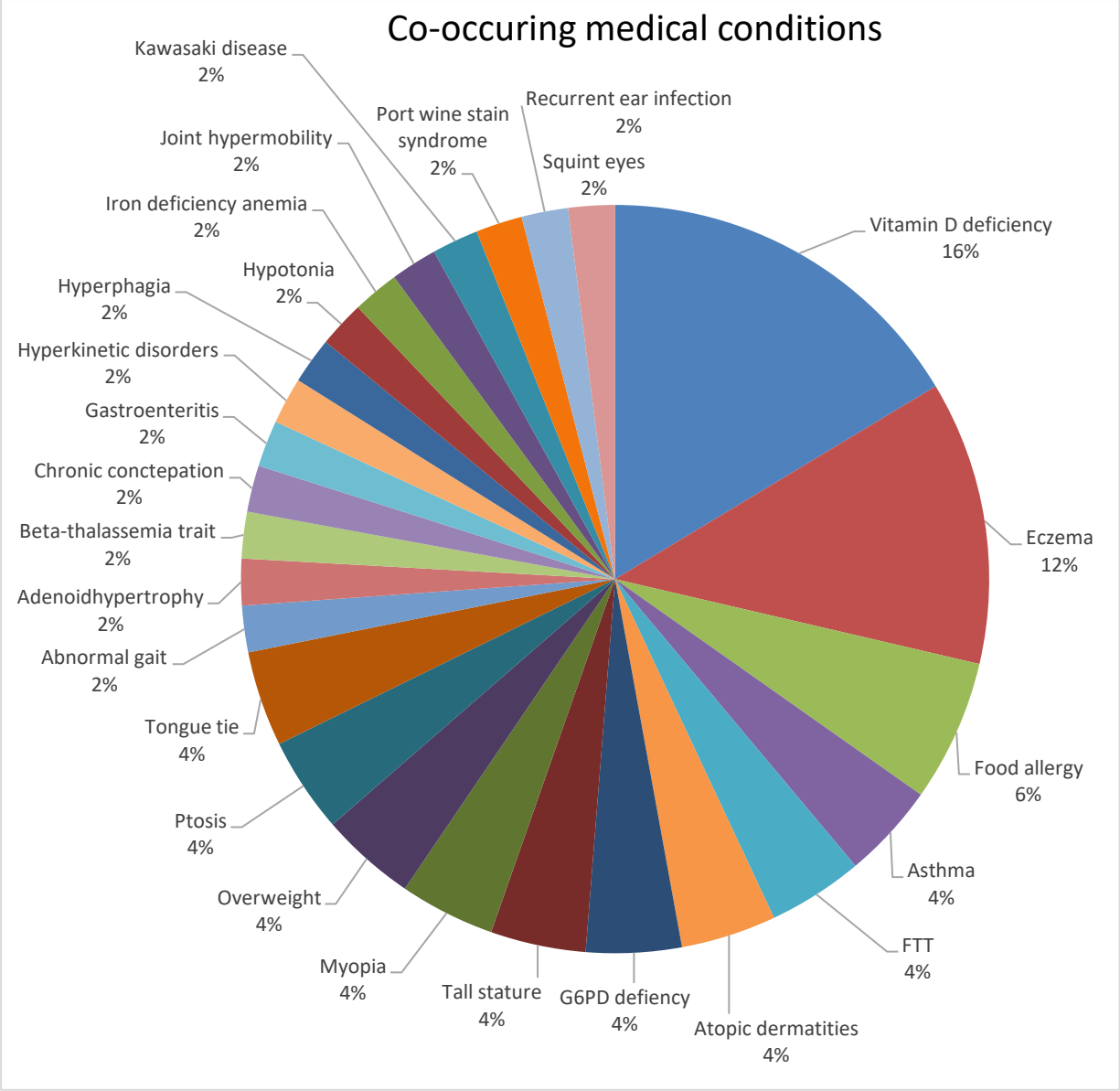

Figure 3. Frequency of 26 identified co-occurring medical conditions

Out of the 276 ASD cases who completed their FXS testing, 270 were studied further by CMA and 115 by WES. Five ASD cases skipped CMA testing and were studied directly by WES. For those 25 patients who had not been tested by FXS testing, 19 were studied directly by CMA, 11 were studied further by WES and 6 were studied directly by WES. Of the total sample, 126 patients completed CMA + WES, 11 did WES only, and 163 CMA only (Figure 4). In conclusion, 276 (91.7%), 289 (96%) and 137 (45.5%) had been tested by FXR, CMA and WES respectively

(Table 9).

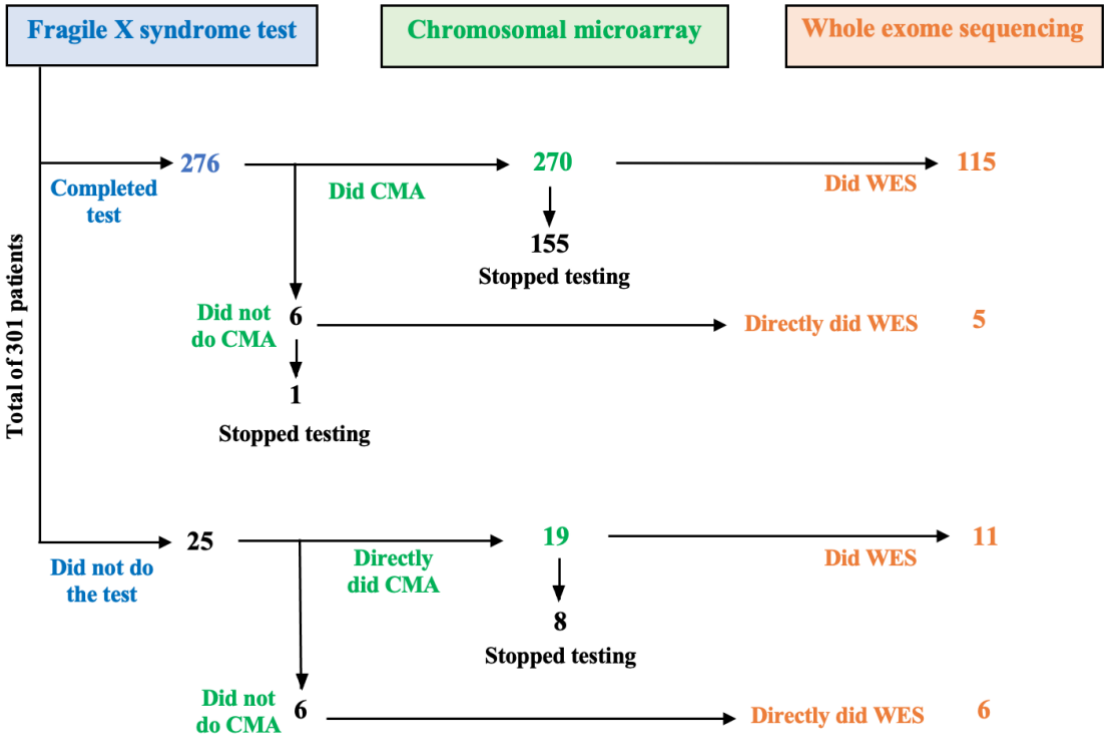

Figure 4. The diagnostic odyssey of the three genetic testing of FXR, CMA, and WES

Of the total sample, 276/301 patients (91.7%) completed FXS testing of them, 272/276 (98.6%) had normal FXS results (CGG repeats 5-44) while 3/276 males had results in the intermediate zone (CGG repeats of 45-54) and one female patient had premutation (CGG repeats of: 55-200).

For CMA, 289/301 (96%) ASD cases had completed the CMA testing, 240/289 (83%) had negative results and 49/289 (17%) patients had positive results. Variants included 10 benign, 42 VUS and 9 pathogenic variants (a total of 61 variants as two patients had two variants each; one had two VUSs and the other had one VUS and one benign). All variant classifications for CMA were reported by the molecular laboratory.

For WES, results of the 137/301 (45.5%) ASD cases who completed the test, 65/137 (47.4%) had negative results and 72/137 (52.6%) had positive results. Variant included, 1 benign, 84 VUS and 22 pathogenic variants (a total of 107 variants, as some patient had more than one variant in different groups, Figure 5).

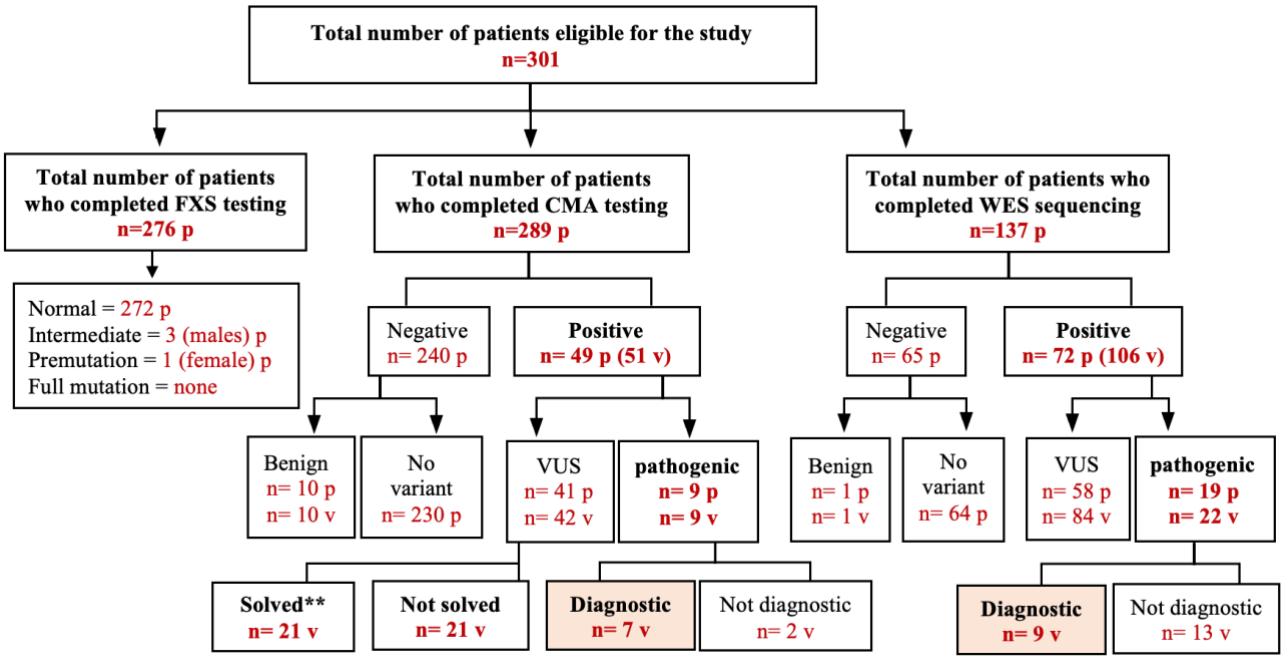

Figure 5. Tests done by the study subjects

v= variant / p= patient / **familial variant inherited from one healthy parent

Of the 137 patients who did WES, only 32 (23.3%) completed reWES (whole exome sequencing reanalysis) after at least one year of the initial WES, however only one patient had change in classification of an earlier reported variants. This patient had benign variant by reWES which was initially reported as VUS in addition, this patient had another VUS variant, so he was still considered as positive WES for statistical analysis purposes.

As not all patient completed CMA and WES, of the 12 patients who did not do CMA, 8 were Qataris and 4 non-Qataris. All patients who did not complete CMA

testing, performed WES directly except for one non-Qatari patient from the ASD complex group who decided to stop at FXS and did not do CMA or WES. A statistical significance was found (p=0.001) between those who completed CMA and nationality (Figure 6) as non-Qatari tend to do CMA more commonly than Qataris. Moreover, significant results (p<0.001) were found between patients who completed WES and who did not as Qatari tend to significantly do WES compared to non-Qatari (Figure 7).

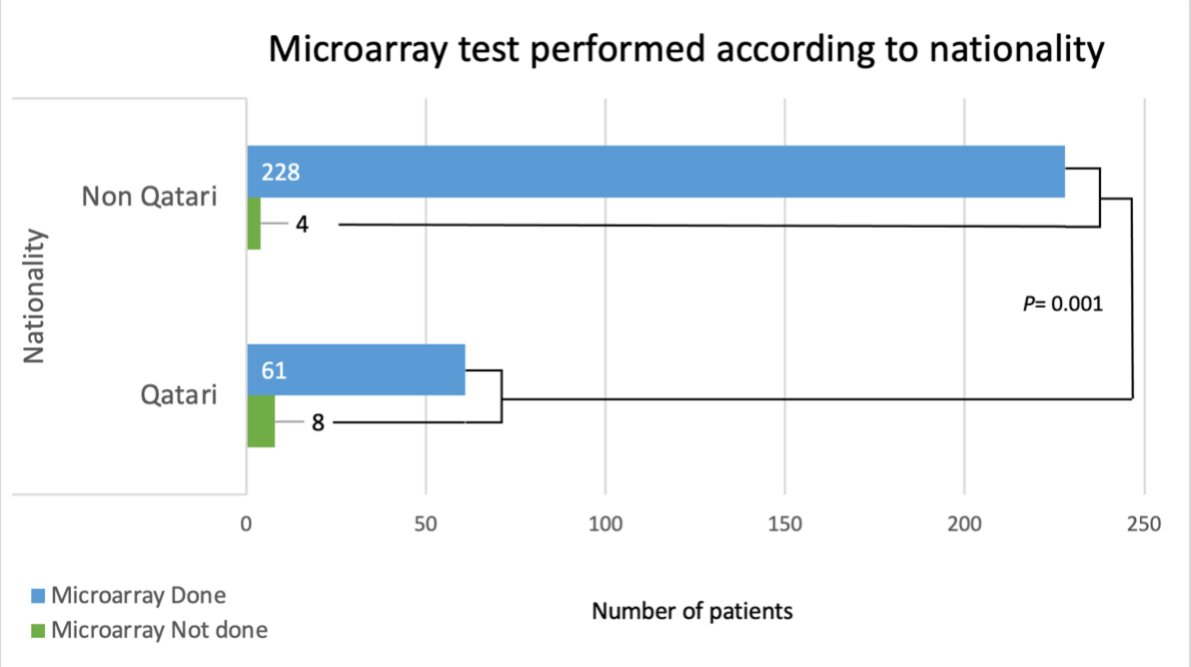

Figure 6. The utilization of the CMA test in Qataris versus non-Qataris ASD patients

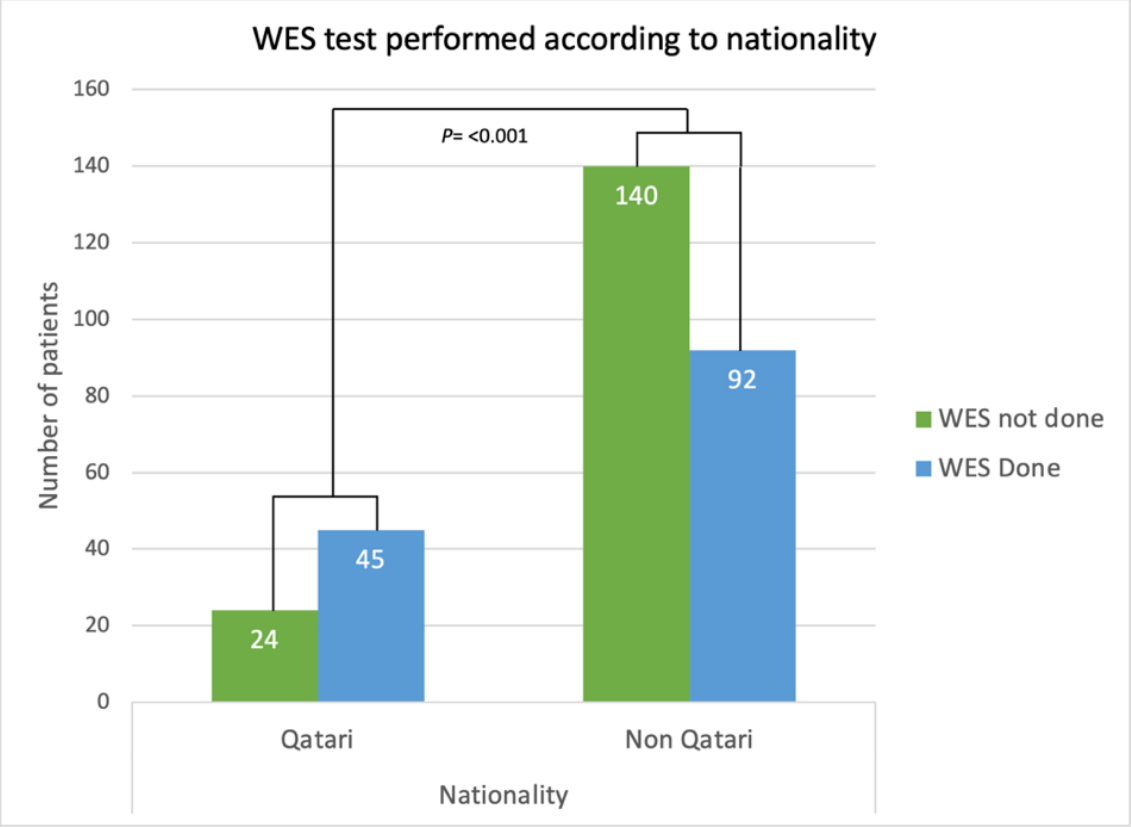

Figure 7. The utilization of WES test in Qatari versus non-Qatari ASD patients

4.2 Diagnostic yield calculation of FXR, CMA and WES

None of ASD cases had a full mutation in *FMR1* gene, only 1 female (0.36%) had a premutation with one allele identified at the lowest limit of the premutation range for trinucleotide CGG repeats (approx. 55 repeats) and the other allele in the normal trinucleotide CGG repeat range (approx. 31 repeats), and 2 (0.7%) had results in the intermediate zone; thus, the diagnostic yield was 0%.

The diagnostic yields for CMA and WES were calculated for those who had positive test results only (Table 10) and variants were considered for diagnostic assessment only if they were classified as pathogenic.

Table 10. Results of Patients Who Completed Genetic Testing

Test results				
FXS	**Positive Classification**	4 patients (1.5%) *		
Total= 276 p		Intermediate	Pre mutation	Full mutation
		3	1 (25%) **	0 (0%) **
	Negative	272 patient (98.5%) *		
CMA	**Negative Classification**	240 (83%) *		
Total= 289 p		Benign	No variant	
Total= 61 v		10 variants (16.4%) **	230 patients	
	Positive Classification	49 patient (17%) *		
		Pathogenic[a]	VUS	
		9 variants (14.7%) **	42 variants (68.9%) **	
WES	**Negative Classification**	65 patient (47.4%) *		
Total= 137 p		Benign	No variant	
Total = 107 v		1 variant (0.9%) **	64 patients	
	Positive Classification	72 patient (52.6%) *		
		Pathogenic[a]	VUS	
		22 variants (20.6%) **	84 variants (78.5%) **	

P: Patient, v: variant
*Percentage within type of test category
**Percentage within total number of variants
a= variants that were considered for diagnostic assessment

4.2.1 CMA diagnostic yield and genetic contribution

On the one hand, 240 out of the 289 (83%) ASD cases who completed CMA testing had negative results (including 10 variants that were likely benign). On the other hand, 49 (17%) of ASD cases had positive results regardless of them being diagnostic or not. No significant difference was identified in receiving negative or positive CMA results amongst the five ASD groups (p=0.146) (Table 11).

Table 11. Patients With Negative/Positive and Diagnostic CMA Results

	Group						
	High Functioning ASD (n=10)	**Non-Verbal ASD (n=105)**	**ASD And ADHD (n=16)**	**ASD Complex (n=129)**	**ASD and DD / ID (n=29)**	**Total (n=289)**	**p-value**
CMA results							
Negative	8 (80.0%)	85 (81.0%)	16 (100.00%)	104 (80.6%)	27 (93.1%)	240 (83.0%)	0.146 [a]
Positive	2 (20.0%)	20 (19.0%)	0 (0.00%)	25 (19.4%)	2 (6.9%)	49 (17.0%)	
CMA diagnostic							
Diagnostic	0 (0.0%)	5 (4.8%)	0 (0.0%)	2 (1.6%)	0 (0.0%)	7 (2.4%)	

	High Functioning ASD (n=10)	Non-Verbal ASD (n=105)	ASD And ADHD (n=16)	ASD Complex (n=129)	ASD and DD / ID (n=29)	Total (n=289)	
% Within CMA diagnostic	0.0%	71.4%	0.0%	28.6%	0.0%	100.0%	0.55[a]
Non-diagnostic	10 (100.0%)	100 (95.2%)	16 (100.0%)	127 (98.4%)	29 (100.0%)	282 (97.6%)	
% Within CMA non-diagnostic	3.50%	35.5%	5.7%	45.0%	10.3%	100.0%	

a= p-value for the 5 groups comparison using Fisher exact test, (p-value is not significant >0.05)

It is worth mentioning that positive results were identified in 25 (19.4%) and 20 (19.0%) of ASD Complex and non-verbal ASD respectively, none of the patients in the other groups received positive CMA.

CMA identified 9 (14.7%) pathogenic variants in known disease-causing regions. The 42 VUSs identified represent 68.9% of the total variants reported, which were divided into two groups: solved (n=21 variants) for those who did parental testing and showed that the variant was inherited from one healthy parent as these CNVs are less likely to be disease causing (maternally inherited n= 13, paternally inherited n=8). Unsolved (n=21 variants) for those who did not complete parental testing as the variant might still be associated with their phenotypes (Table S1 and Table S2). Of the patients who did parental testing all the variants were inherited from one healthy parent, and none were reported as *de novo* which also supports their less likely association with the disease. Benign variants were considered negative and not disease-causing as reported by the laboratory (Table S3).

Only pathogenic variants (n=9, 14.7%) were eligible for diagnostic assessment in case they match the clinical phenotype of ASD in the patient otherwise it was considered pathogenic non-diagnostic (Table 12). Two variants were considered non-diagnostic as they were associated with diseases other than ASD, one variant

identified in a male patient (deletion of cytogenetic band Xq13.1 that contains *EDA* gene) was associated with X-linked recessive Ectodermal dysplasia type 1 [160] and the other variant identified in a female patient (deletion of cytogenetic band 17p12) was associated with autosomal dominant hereditary neuropathy with liability to pressure palsies (HNPP) [161]. The other seven identified variants were considered diagnostic as all of them were associated with phenotypes such as ASD, developmental delay and other behavioral problems. Of the total diagnostic variants, five were identified in males while two were identified in females with size variations form 31 kb to 8 Mb, five of the diagnostic CNVs were deletions compared to two duplications. All variants were reported earlier in other populations. Three out of the seven pathogenic diagnostic variants were inherited from a healthy parent however low penetrance cannot be excluded and thus cannot rule out the pathogenicity of the variants.

In summary, out of the total sample size (n=289), the overall CMA diagnostic yield is 2.4% identified in two groups only; non-verbal ASD with 71.4% of the total CMA diagnostic results followed by ASD complex with 28.6%. None of the patients in high functioning group, ASD and ADHD groups and ASD and DD/ID had diagnostic CMA results, however, no statistical significance was found between the five groups (p=0.55) (Table 11). Consanguinity was not statistically significant between those who received diagnostic results and those with non-diagnostic/negative CMA (p=0.334).

Table 12. Pathogenic (diagnostic and non-diagnostic) Variants Reported From CMA Study

RN	Group	Gender	Chromosomal Region	Genomic Coordinates	Del/Dup	Size/ Gene[a]	Inheritance	Ref
28	ASD complex	Male	4q32.1q32.3	(157,216,436-165,463,766)	del	~8 Mb	*De novo*	[162]

RN	Group	Gender	Chromosomal Region	Genomic Coordinates	Del/Dup	Size/ Gene[a]	Inheritance	Ref
38	ASD complex	Female	1q41	(218,416,890-218,559,329)	dup	~142 kb *TGFB2*	ND	[163, 164]
59	Non-verbal ASD	Male	Xq13.1**	(69,002,654-69,034,183)	del	~31 kb *EDA*	Maternal	[160]
86	Non-verbal ASD	Female	17p12**	(14,111,972-15,442,119)	del	~1.3 Mb *PMP22*	ND	[161]
148	Non-verbal ASD	Male	16p11.2	(29,620,717-30,190,593)	del	~570 kb	*De novo*	[88, 165]
571	Non-verbal ASD	Female	14q11.2	(21,862,597-21,981,371)	del	~118 kb *CHD8*	Not paternal Maternal ND*	[166-168]
607	Non-verbal ASD	Male	Xq27.3q28	(146,959,715-147,195,958)	del	~236 kb *FMR1*	Maternal	[169]
628	Non-verbal ASD	Male	7q35	(146,141,677-146,289,810)	del	~148 kb *CNTNAP2*	Paternal	[170]
650	Non-verbal ASD	Male	16p11.2	(31,253,845-31,934,803)	dup	~681 kb	ND	[88, 165]

RN= Research number/ Del= deletion/ dup= duplication/ kb= kilobase/ Mb= mega base/ ND= not done/ a= genes within this region according to the laboratory reporting/ *only father did familial testing and came negative, the mother did not do the test / **non-diagnostic results/ ref= reference

4.2.2 WES diagnostic yield and genetic contribution

Of the 137 patients who completed WES, more than half (52.60%) received positive results with a significant *p*-value (0.022) reported amongst groups. Post hoc analysis showed that ASD and DD/ID group had a significant *p*-value (0.003) compared to all other groups as the results of this group showed 19 positive cases out of the 24 patients tested, representing 79.2% of the total group (Table 13). Although the highest number of positive results was reported in ASD complex group however according to group size (n=62) these positive results represent only 40.3% of the total group.

Table 13. Summary of WES Positive/ Negative Results Frequency and Diagnostic Yield in Each Group

	Group						
	High Functioning ASD (n=6)	**Non-Verbal ASD (n=38)**	**ASD And ADHD (n=7)**	**ASD Complex (n=62)**	**ASD and DD / ID (n=24)**	**Total (n=137)**	**p-value**
WES results							

WES results	High Functioning ASD (n=6)	Non-Verbal ASD (n=38)	ASD And ADHD (n=7)	ASD Complex (n=62)	ASD and DD / ID (n=24)	Total (n=137)	
Negative	3 (50.00%)	17 (44.70%)	3 (42.90%)	37 (59.70%)	5 (20.80%)	65 (47.40%)	0.022 [a]
Positive	3 (50.00%)	21 (55.30%)	4 (57.10%)	25 (40.30%)	19 (79.20%)	72 (52.60%)	
p-value [b]	0.92	0.68	0.84	0.009	0.003 [c]		
WES diagnostic							
Diagnostic	0 (0.0%)	2 (5.3%)	0 (0.0%)	1 (1.6%)	6 (25.0%)	9 (6.6%)	0.01 [a]
% Within WES diagnostic	0.0%	22.2%	0.0%	11.1%	66.7%	100.0%	
Non-diagnostic	6 (100.0%)	36 (94.7%)	7 (100.0%)	61 (98.4%)	18 (75.0%)	128 (93.4%)	
% Within WES non-diagnostic	4.7%	28.1%	5.5%	47.7%	14.1%	100.0%	
p-value [b]	0.48	0.68	0.48	0.03	0.0001[c]		

a= p-value for the 5 groups comparison using fisher exact test, (p-value is significant <0.05)
b= post hoc comparison conducted using fisher exact test after Bonferroni correction (p-value <0.005)
c= statistically significant p-value

Of the 22 pathogenic variants, only 9 were considered diagnostic and were identified in 9 diffrent patients while the other 13 variants (identified in 10 patients) were considered non-diagnostic (Table 14, Table S5). For those with non-diagnostic variants, three patients had positive WES results due to reported pathogenic ACMG secondary findings associated with hereditary breast and ovarian cancer, arrhythmogenic right ventricular cardiomyopathy, and familial hypercholesterolemia. Three patients were only carriers for autosomal recessive conditions (early onset epileptic encephalopathy, tay sachs disease and maple syrup urine disease). One patient had a pathogenic mitochondrial variant however the reported heteroplasmic level was very low (2%) and not enough to cause disease. The other associated diseases are not linked to ASD which include, thiamine-responsive megaloblastic anemia syndrome, oculocutaneous albinism type 1, autosomal dominant multiple epiphyseal dysplasia, hemoglobinopathies, and seizures related disorders (Table S5).

Out of the 137 patients, the total sample WES diagnostic yield identified is 6.6% with the highest yield reported in ASD and DD/ID group as it was able to

genetically diagnose 6 patients representing 25% of the total sample who tested positive and 66.7% of the total diagnostic yield among all five groups. A statistical significance was reported among all groups and post hoc analysis showed that ASD and DD/ID had a statistically significant *p* value of 0.0001 indicating that this groups had significantly higher diagnostic yield compared to other groups (Table 11). No diagnostic variants were identified in either high functioning group or ASD and ADHD groups.

Of the 9 diagnostic results, 6 (66.6%) were identified in males and 3 (33.3%) in females. The most common mode of inhertance reported in our sample is X linked (5 variants; 55.6%) followed by autosomal dominant (2 variants; 33.3%) and autosomal recessive (1 variant; 11.1%). Five variants were inherited as *de novo* (three dominant and two X linked) and two x-linked variants identified in males were inherited from healthy mothers, while the other two variants being of unknown inheritance (one X linked and one autosomal reccisive) as the parents were not included in the test. Five variants in five different genes were labeled "novel" as none of these variants had been reported in the literature (Table 14). Consangunity was not statistically signifcant between those who received diagnostic results and those with non-diagnostic/negative WES (*p*=0.347).

Table 14. Diagnostic pathogenic variants identified through WES

RN	Group	Gender	Gene	Disease	MOH	cDNA Level	Protein Level	Zygosity	Inheritance*	Ref
73	ASD and DD/ID	Male	*TUSC3*	TUSC3 related disorder	AR	c.1028 G>C	p.S343T	HM	Unknown	[15]
286	Non-verbal ASD	Female	*MECP2**	MECP2 related disorder / Rett syndrome	X linked	Deletion of exon 1	/	HT	Unknown	[171]
411	**Non-verbal ASD**	**Male**	***CUL4B***	**CUL4B related disorder**	**X linked**	**c.2554 G>A**	**p.A852T**	**HMi**	***De novo***	**Novel**
646	**ASD and DD/ID**	**Male**	***NAA10***	**NAA10 related disorder**	**X linked**	**c.49 T>G**	**p.C17G**	**HMi**	**Mother**	**Novel**

RN	Group	Gender	Gene	Disease	MOH	cDNA Level	Protein Level	Zygosity	Inheritance*	Ref
666	ASD and DD/ID	Female	*SHANK3*	SHANK3 related	AD	c.3679dupG	p.A1227Gfs X69	HT	*De novo*	[172]
947	**ASD complex**	**Male**	***ATRX***	**ATRX related disorder**	**X linked**	**c.559 T>C**	**p.Y187H**	**HMi**	**Mother**	**Novel**
836	**ASD and DD/ID**	**Male**	***ZNF462***	**ZNF462 related disorder**	**AD**	**c.1351 C>T**	**p.R451X**	**HT**	***De novo***	**Novel**
64	ASD and DD/ID	Male	*POGZ*	White Sutton syndrome	AD	c.3041delA	p.Q1014Rfs X5	HT	*De novo*	[15]
995	**ASD and DD/ID**	**Female**	***DDX3X***	**DDX3X related disorder**	**X linked**	**c.112 T>C**	**p.Y38H**	**HT**	***De novo***	**Novel**

RN= research number/ ref= reference/ *Inherited from which parent/ AR= autosomal recessive/ AD=autosomal dominant/ HM= homozygous/ HT= heterozygous/ HMi= hemizygous

Table 15. Pathogenic Variants Analysis in Diagnostic Cases

Group	Gene		
Previously reported diagnostic variants	**Autosomal recessive (AR)**	**Autosomal dominant (AD)**	**X-linked**
	TUSC3	*SHANK3, 2, POGZ*	*MECP2,*
Novel diagnostic variants	**Autosomal recessive (AR)**	**Autosomal dominant (AD)**	**X-linked**
	/	*ZNF462*	*CUL4B, NAA10, ATRX, DDX3X*

The clinical characteristics of the patients with diagnostic WES results are summarized in Table 16. Five of the patients were Qataris (55.5%) while the other four were from different nationalities (44.5%). Of the Qatari patients, four had consanguineous parents (three are first cousins and one are from the same family but far relatives, 80%), however, the variants identified in these patients were two heterozygous variants (one *de novo* and one of unknown inheritance), one homozygous (unknown inheritance) and one hemizygous identified in male patient (*de novo*). The one Qatari patient with non-consanguineous parents had a heterozygous variant inherited as *de novo*. Of the other nationalities (Indian, Omani, Filipino, and Jordanian) only the Omani patient had consanguineous parents but had heterozygous *de novo* variant. The Indian and Filipino patients (both males) had hemizygous variants inhered from healthy mothers, while the Jordanian patient had

heterozygous *de novo* variant. All patients had unremarkable pregnancy/delivery and normal hearing and vision with varying other clinical presentations.

Table 16. Clinical Characteristics of Patients With Pathogenic Diagnostic WES Results

Research number	Gender/ Nationality	Clinical presentation	Consanguinity/ family history
73	Male/ Qatari The patient was seen in the genetics clinic at 10 years old	- Pregnancy/Delivery: unremarkable - Development: normal gross motor - Intellectual disability - Speech delay - Hyperactivity - Learning Disability - The patient has failure to thrive (FTT) - Normal Hearing - Normal Vision	- Parents are consanguineous: same family - Older Brother with autism - WES proband/ no segregation results
286	Female/ Qatari The patient was seen in the genetics clinic at 2 years old	- Pregnancy/Delivery: unremarkable - Development: normal gross motor - On Examination: within normal Cafe au lait spot on left side of chest wall 2 cm, no other patches - Severe receptive and severe expressive speech and language delay - Normal Hearing - Normal Vision - Clinical recommendations after WES results: referred to neurology	- Parents are consanguineous: first cousins - No family history
411	Male/Qatari The patient was seen in the genetics clinic at 4 years old	- Pregnancy/Delivery: unremarkable - Development: normal gross motor - He has distal penile hypospadias and small penis. - He has asthma - Severe receptive and severe expressive speech and language delay - Normal Hearing - Normal Vision - Clinical recommendations after WES results: referred to neurology and endocrinology	- Parents are consanguineous: first cousins - Family history: older sister with speech delay, learning difficulties and hearing impairment - WES trio/ negative segregation in older sister
646	Male/ Indian The patient was seen in the genetics clinic at 3 years old	- Pregnancy: by in vitro fertilization, part of twin, due to infertility - Developmental delay - Speech delay - Normal Hearing - Normal Vision	- Parents are not consanguineous - Family history: Twin brother with autism. - WES trio/ segregation proved the presence of the variant in the affected twin brother

Research number	Gender/ Nationality	Clinical presentation	Consanguinity/ family history
666	Female/ Omani The patient was seen in the genetics clinic at 8 years old	- Pregnancy/Delivery: unremarkable - Development: normal gross motor - Intellectual disability - Attending special needs schools - Normal Hearing - Normal Vision	- Parents are consanguineous: first cousins - No family history
947	Male/ Filipino The patient was seen in the genetics clinic at 8 years old	- Pregnancy/Delivery: unremarkable - Development: normal gross motor - Mild to moderate receptive and expressive speech and language regression and disorder - Sensory processing disorder - ADHD - History Kawasaki disease at 1.5 years of age, diagnosed and treated in Philippines. - Normal Hearing - Normal Vision	- Parents are not consanguineous - Family history: ASD in a maternal first cousin once removed / ADHD in a paternal second cousin - No segregation
836	Male/ Qatari The patient was first in the genetics clinic at 6 years old	- Pregnancy/Delivery: unremarkable - Development: normal gross motor - Developmental delay (mainly speech and cognition) - Social communication difficulties - Bilateral congenital ptosis - Normal Hearing	- Parents are consanguineous: first cousins - No family history
64	Male/ Qatari The patient was seen in the genetics clinic at 17 years old	- Pregnancy/Delivery: unremarkable - Development: normal gross motor - learning problem, attending special need school - Mild intellectual disability - Asthma - Prominent incisors teeth - Truncal obesity - Normal Hearing - Normal Vision	- Parents are not consanguineous - No family history
995	Female/ Jordanian The patient was seen in the genetics clinic at 6 years old	- Pregnancy/Delivery: unremarkable - Development: delayed walking - Speech delay - Intellectual disability - History of repeated elbow dislocation while young till the age of two years - Disturbed bowel motion. - Small sized head was reported prenatally - Normal brain MRI - Normal Hearing - Normal Vision	- Parents are not consanguineous - No family history

The 84 VUSs identified in 58 patients were reported in 76 different genes of them 6 were reported in mitochondrial DNA (Table 17). Of the total reported variants 13 were reported as *de novo* while 14 were of unknown origin as no parental study

was done, the other 57 were inherited from either one healthy parent or from both parents in some cases of homozygous variant.

Table 17. Genetic Contribution of The Identified VUS in The Studied Cohort

Group	Gene			
Nuclear DNA	**Autosomal recessive (AR)**	**Autosomal dominant (AD)**	**X-linked**	**No diseases are currently described**
	CC2D2A, SBF-1, DPYD, NDST1, NLRP12*, ALG8, NARS2, MPDZ, CEP290*, DST*, MYO6, RARS**	*DEPDC5, DYRK1A, KCNQ2, SOS2, SLC9A9, NTNG1, TSC2, SLIT3, NLRP12, PDGFRB, MITF, CHD8, CTNND2, KCNJ6*	*ZNF711, ATP6AP1, ALG13, BRWD3, HUWE1, ARHGEF6, AMER1, FLNA, FGD1, LAMP2, MECP2, TAF1, MED12, ZNF711*	*ATAD3C*, SH2B3*, SMPD4, MED24, ABCA2, FAM46A, PAPPA2, NRCAM, SLC2A6, OSBP, AMOT, CADPS2*, H2BFM, RYR3*, UPF1, DIPK2B, ANKHD1, DLG1, LRP1, WDFY3, SLC18A1, PRRC2B, ATP10A, LINGO1, ZFHX4, AHNAK, WWOX, TBL1X, EIF3L, PUM2*
Mitochondrial DNA	**Homoplasmic (HMp)**		**Heteroplasmic (HTp)**	
	MT-CO3, MT-ND4, MT-TQ, MT-ND6		*MT-ND6, MT-CO3*	

*Compound heterozygous variants were inherited in these genes

None of the patients in our cohort had diagnostic results from both CMA and WES. Of the 7 patients who had diagnostic results by CMA, none of them continued WES testing except for one patient (research number 28) who completed WES and received VUS in mitochondrial gene (Table S4). Of the 9 patients who had diagnostic WES results, 22.2% (n=2) did not do CMA, while 66.6% (n=6) had negative CMA results, except for one patient (11.2%) (research number 411) who received VUS by CMA (22q11.21 dup) inherited from a healthy father.

4.2.2.1 WES clinical reassessment

WES was not diagnostic for 128/137 (93.4%) of patients, with no reportable variants or where the reported variant did not explain the proband's phenotype. Nevertheless, clinical reanalysis of the VUSs reported in 58 patients through clinical

correlation performed by a group of expert clinical geneticists, genetic counselors, and clinical laboratory scientists revealed likely diagnostic results in 8 patients, corresponding to 13.8% of reanalysis cases. These results might increase the diagnostic yield to 12.4% (17/137) however more evidence is needed to support the pathogenicity and the link of these likely associated variants with the phenotype (Table S4).

Chapter 5: Discussion

ASD is one of the most frequently studied neurodevelopmental disorders, especially with the dramatic increase in its prevalence in the last decade that can be explained by the advancement of technology and expansion of diagnostic criteria of the disease [173]. The diagnostic yield of genetic testing in ASD cases is poorly studied in Arab countries. This study investigated the genetics of ASD in Qatar and compared the diagnostic yield of different genetic tests available in Qatar.

In the current study, we focused on the diagnostic yield of non-syndromic ASD by excluding patients who have other neurological disorders and brain malformation as these comorbidities overlap with the genetics of ASD, which will result in difficulties correlating the genetic diagnosis with the phenotype [174] and will overlap with the understanding of the underlying molecular/genetic mechanism of the disease [175]. Moreover, the presence of structural congenital anomalies and dysmorphic features will increase the risk of ASD-associated syndromes and secondary ASD [176]. In our study, we divided the patients into five different groups based on their behavioral clinical symptoms associated with ASD. Patients were divided based on the most common behavioral comorbidities into; high functioning ASD for those who had problems with communication only but had normal cognitive and functional skills [177], non-verbal ASD for those with severe speech delay and language difficulties [178], ASD with ADHD [179] and complex groups for those with more than one comorbidity. Patients with DD/ID were defined as a separate group because DD/ID is considered as a neurodevelopmental disordered (NDD) with very increased prevalence in ASD, and to date, only ASD and DD/ID have recommendations of genetic testing [176]. Other studies followed different classification approach, one study subclassified their patients based on their most

frequent accompanying signs and symptoms, including epilepsy; micro/macrocephaly, and syndromic forms [180]. Furthermore, one study divided their ASD patients based on the presence of major congenital abnormalities and minor physical anomalies [13]. To our knowledge, none of the published papers followed a similar approach to ours, which we believe is the best approach to get accurate diagnostic yields without the presence of other underlying medical conditions such as seizures or congenital anomalies which can bias the real diagnostic yield related to ASD.

The male to female ratio identified in our sample was reported as 3.6:1 of the total sample size (n=301) with a ratio ranging from 2.6:1 – 16:1 between groups (Table 9) which is similar to the estimate of male to female ratio in ASD cases (3:1) reported by a systematic review published in 2017 [5]. Our results are also concordant with a previous study of a large ASD sample in Qatar which also showed a ratio of 4:1 [181]. In our cohort, 30.6% had consanguineous parents which is concordant to a previous study performed on ASD patients in Qatar in 2017 that reported parental consanguinity of 40% among ASD patients however this study showed that the effect of consanguinity as a risk factor was not found to be significant [102]. A study published in 2015, explored the association of consanguinity and the development of ASD in the Indian population and results showed increased rates of consanguinity in the ASD group compared to controls [182]. Moreover, a recent study by Guisso et al. (2018) concluded that consanguinity is a risk factor for the development of ASD in a Lebanese consanguineous population [181]. The relationship between consanguinity and the possibility of receiving positive diagnostic results from CMA was studied in highly consanguineous Omani population with reported parental consanguinity of 31%. No statistical significance (p=0.663) was found between parental consanguinity

and receiving diagnostic CMA results [183] these results are similar to our study that reported no significance between receiving diagnostic either CMA or WES and having consanguineous parent (p=0.334, p=0.347 respectively). Due to the increased consanguinity reported in Qatar, we expected higher rates of homozygous variants associated with ASD reported by WES however, most of the pathogenic variants were inherited in heterozygous status (55.5%) followed by hemizygous (33.3%) and only one patient of consanguineous parents had homozygous variant (11.2%). However, 16.7% (14/84 variant) of the VUSs reported were inhered as homozygous and that can be explained by the increased rates of consanguinity in the sample as 71.4% of the patients (10/14) who had homozygous VUSs are from consanguineous parents while the other 28.6% did not do parental testing, thus we expect higher consanguinity rates within these groups. Although these VUS variants are not classified as pathogenic, we were able to clinically link some of them to the phenotype of the patient and thus they should be prioritized in a further investigation for potential reclassification perhaps by conduction functional in vitro and /or in vivo studies.

We explored the relationship between nationality and genetic testing, a statistical significance was found between those who have received WES testing and those who did not based on nationality (p=<0.001). Although testing was offered to all, Qatari patients tend to do WES more frequently than non-Qataris and that can be explained by the high cost of the test [184]. Moreover, non-Qataris were more likely to start with CMA as it is done in a local laboratory with no charge compared to Qataris who skip CMA and move directly to WES (p=0.001).

5.1 FXS diagnostic yield

FXS due to *FMR1* expansion is considered one of the most common forms of monogenic ASD [123]. In our sample no diagnostic yield was identified as none of

the patients had a full mutation, similar results were reported in exploring the diagnostic yield of FXS in different neurodevelopmental disorders including ASD [180]. This might be explained by the fact that the majority of FXS patients express clinical features at a young age and are usually diagnosed with FXS first then ASD and 96% of them usually have a positive family history of the disease [185]. Recent evidence supports that the frequency of FXS has been overestimated over time, with most patients having either compatible clinical features or family history suggestive of this disorder [185]. This proposed that FXS should be considered a second-tier testing in neurodevelopmental disorders including ASD in case of negative clinical features and family history [185]. Our results are concordant with a study conducted by Weinstein. V *et al.* (2017) showed 0% diagnostic yield of FXS testing in ASD patients [186].

Our study reported one female patient who had FXS pre-mutation in one allele inherited from a healthy mother. A study found an increased rate of ASD in pre-mutation siblings of an individual with FXS compared with noncarrier siblings [187]. In a large survey of families, 19% of males and 1% of females with the pre-mutation had a diagnosis of ASD [188]. Similarly, in a direct-screening study, 14% of boys and 5% of girls with the pre-mutation were found to have ASD. This is an elevated risk relative to the general population, however, cannot be considered as diagnostic [127].

5.2 CMA diagnostic yield

In our study, we explored the diagnostic yield of CMA and WES in the five defined ASD groups considering in a first step the possibility of receiving positive (Likely pathogenic/ abnormal and VUS) or negative results (Benign/Likely Benign/ no variant reported). This approach was followed because the classification of VUS remains a challenge, 68.9% of the total identified CNVs were reported as VUS in our

samples which are considered high compared to previous studies that identified only 8.3% [189] and 14% [190]. Despite their uncertainty, VUS might be a good candidate marker for rare developmental disorders including ASD thus they were considered as an indication of positive results. However, no statistically significant difference between the five groups and CMA positive or negative results was identified (p=0.146). Parental testing is recommended after identifying VUS in CMA [189] thus our findings were divided into two groups solved and unsolved based on parental testing. Although this might help in explaining the uncertainty of results however most CNVs with different locations, did not show primary apparent clues suggestive of further genotype-phenotype correlation analysis, even within the same family.

In our approach, the diagnostic yield has been evaluated based on the positive results that include pathogenic variants which were reported to be related to ASD and its associated phenotypes only and we have named these variants as diagnostic variants. Of the total patients who completed CMA (n=289) the diagnostic yield reported is 2.4% identified in two groups only: non-verbal ASD and ASD complex. None of the patients in high functioning group, ASD and ADHD groups and ASD and DD/ID had diagnostic CMA results, however, no statistical significance was found between the five groups due to the low diagnostic yield identified in the whole sample (p=0.55). The overall diagnostic yield identified in our study is low compared to other studies that reported 9.3% [13] and 11.8% [191]. These differences might be attributed to multiple factors such as the criteria used for patient selection being our cohort characterized by a high clinical homogeneity compared to other studies where the genetic contribution to the phenotype is expected to vary widely; the sample size studied; the timing of the study and therefore, the knowledge about the CNVs; the variant classification criteria as well as the CMA methods used in other studies and

their resolution. Arrays with higher probe densities generally lead to an increase in the detection yield that is often accompanied by an associated increase in the number of VUS that are detected [185]. Studies with higher CMA diagnostic yield compared to ours had heterogenous samples where patients had clinical features other than ASD such as seizures, dysmorphic features, and congenital anomalies which increases the chance of receiving diagnostic results from CMA regardless of the diagnosis of ASD [192]. Indeed, several studies have evidenced the predictor effect of congenital malformations, facial dysmorphic features, and others, in finding a pathogenic variant by CMA [193-196].

Of the diagnostic CNVs identified, one patient had *de novo* 4q32.1q32.3 deletion which is considered rare and not linked to syndromic ASD however has been reported previously in children with mild dysmorphic features, late presentation of learning difficulties, and behavioral problems [162]. Another patient had 1q41 deletion that includes the gene *TGFB2* associated with Loeys-Dietz syndrome (LDS) [163] although this syndrome is mainly related to cardiovascular and musculoskeletal disorders however patients with LDS report at least one neurodevelopmental problem including ASD in most cases [164]. Two patients had 16p11.2 deletion syndrome which is one of the well-studied syndromes and considered as a recurrent finding in patients with developmental delay, intellectual disability, and/or ASD [88]. This region has been characterized as autism susceptibility in up to 1% of ASD patients [165]. One patient had 14q11.2 deletion which causes partial loss of *CHD8* gene. This CNV and mutations in this gene have been extensively reported in ASD cases [166-168]. In addition, one patient had 7q35 deletion which may result in disruption of *CNTNAP2* gene. Disruption of this gene has been reported in children with autism and speech delay [170]. Another patient was able to receive FXS diagnosis by CMA as

deletion was found in cytogenetic band Xq27.3q28, with an approximate size of 236 kb. This deletion contains the *FMR1* gene associated with Fragile X syndrome [169]. Although the most common cause of fragile X syndrome is an expansion of a CGG trinucleotide repeat in the 5′UTR of *FMR1*. This expansion leads to transcriptional silencing of the gene. However, other mutational mechanisms, such as deletions of *FMR1*, also cause fragile X syndrome [197]. Interestingly, this patient had done FXS CGG repeats testing and received negative results as the CGG trinucleotide repeat is in the normal range (approx. 30) according to the molecular laboratory.

Although no statistical significance was found between groups further studies are needed for high functioning group, ASD and ADHD groups and ASD and DD/ID as these groups might have lower chances of receiving diagnostic results from CMA compared to more complex phenotypes. A recent study by Lopez (2021) et al, reported a similar diagnostic yield of 2.9% (identified in 9 out of 307 ASD cases). Differences in diagnostic yield is also related to the study design, as some studies that reported high diagnostic yield used research CMA while those reported similar results to our used retrospective approach of clinical CMA. This is also supported by a study conducted on ASD cases using different CMA procedures, results showed that research CMA had higher diagnostic yield compared to clinical CMA [13].

5.3 WES diagnostic yield

Like CMA, the comparison of positive/negative WES results in the different ASD groups was explored and a statistically significant difference in the number of positive results was found between ASD and DD/ID group compared to other groups (p=0.003). Moreover, significance was also observed in diagnostic yield (p=0.0001) (pathogenic diagnostic variants) for the same group compared to others. This can be explained by the fact that patients with DD/ID tend to have higher diagnostic yield by

WES compared to other neurodevelopmental disorders as previously reported [198, 199]. The total reported WES diagnostic yield of the sample is 6.6% (9 patients) with 66.7% of the patients being from the ASD and DD/ID group. A higher yield was reported in a previous study (25.8%) however this can be explained by the differences in clinical characteristics of the studied sample [200]. One study reported that lower diagnostic yield of clinical WES is usually observed in isolated ASD cases compared to those with associated phenotypes such as epilepsy, microcephaly/macrocephaly, and syndromic ASD which can explain the findings of our study compared to other studies [180]. Another explanation is reported in the same later reference work that demonstrated the possible overestimation of the whole exome sequencing diagnostic yield due to a bias in the selection of samples for WES. While compared to our samples, WES was the choice of the patient.

Similar to CMA majority of variants identified by WES were VUS (78.5%), an observed increase in studies that reported VUS, starting at around 20% of all reported variants in 2014 and reaching 70% in 2020. A recent meta-analysis of the clinical sequencing yield in epilepsy, autism spectrum disorder, and intellectual disability reported that the number of studies reporting VUS has increased significantly after the introduction of the ACMG guidelines as only two studies reported VUS before 2016, whereas 45 studies reported VUS from 2016 to 2020 [12].

Of the diagnostic genes identified four variants were reported earlier as one of the patients in our study received a diagnostic result by WES due to the extra recommendations from the physician to do the deletion/duplication analysis of *MECP2* as the case was suspected Rett syndrome. The results came positive for deletion of exon1 confirming the diagnose of Rett syndrome in this female patient. The prevalence of ASD is estimated to be 60% among Rett syndrome patients **(Table**

5) and it is recommended to screen for it in all ASD-affected females according to the ACMG guidelines [133].

The other three variants in our cohort were reported in one Qatari patient from consanguineous parents who had a homozygous variant in *TUSC3* gene (c.1028G>C) which has been reported in association with autosomal recessive syndromic and non-syndromic forms of intellectual disability that manifest during early childhood [201]. Interestingly, a recent case report showed that c.1028G>C variant in *TUSC3* gene has been reported in two Qatari sisters who had severe speech delay, severe aggressive behaviors, severe impulsivity, hyperactivity, obsessive behaviors, and emotional dysregulation [202]. Another patient had a heterozygous *de novo* variant in *SHANK3* gene (c.3679dupG) associated with Phelan-McDermid syndrome characterized by neonatal hypotonia, global developmental delay, absent to severely delayed speech, normal to accelerated growth, autistic behavior, and minor dysmorphic facial features [203]. This variant has been reported in two male siblings with ASD with presumed germline mosaicism in their mother [172].

De novo pathogenic variant (c.3041delA) in *POGZ* was reported in male patient which is observed in White–Sutton syndrome a neurodevelopmental syndrome associated with ASD with or without intellectual disability [204] this gene has been widely studied and strongly related to ASD. This variant had been previously found in a Qatari cohort of individuals with neurocognitive phenotypes [15]. In our cohort another patient had duplication of 1q21.3 which cause partial duplication of the *POGZ* gene however this CNV was classified as VUS and was inherited from a healthy mother (Table S2).

Of the five novel variants identified, one patent had hemizygous pathogenic variants in *CUL4B* gene (c.2554 G>A) which cause a syndromic form of X-linked

intellectual disability [205]. One hemizygous pathogenic variant was reported in *NAA10* gene (c.49 T>G) in a male patient that causes X-linked NAT deficiency, which results in intellectual disability, postnatal growth delay, severe microcephaly, recurrent infections, hypotonia, and dysmorphic features [206]. One patient had hemizygous pathogenic variants in *ATRX* gene (c.559 T>C) that causes alpha-thalassemia X-linked intellectual disability (ATRX) syndrome. Newly described variants (p.T1621M) in this gene has been also linked to ASD [207]. One male had a heterozygous diagnostic variant (c.1351) in *ZNF462* gene associated with Weiss-Kruszka syndrome, an autosomal dominant disorder characterized by features including developmental delay and ASD [208]. The last patient was a female with *de novo* heterozygous *DDX3X* variant (c.112 T>C), pathogenic variants in this gene have been strongly linked to ASD, ADHD and ID especially in females [209, 210]. This gene is known to escape X inactivation thus considered disease causing in heterozygous status in females [211]. Although none of these variants has been reported earlier to the best of our knowledge, the association between the reported genes and ASD related phenotypes has been well established.

As expected, whole exome sequencing resulted with the highest diagnostic yield compared to other first-tier tests in the genetic diagnosis of ASD cases. This study supports that WES should be considered as the first-tier test in the genetic diagnosis of ASD since a greater diagnostic yield was observed when compared to either CMA or FXS testing. Specifically, WES identified 6.6% of causative variants in ASD patients whereas diagnostic yield of CMA and FMR1 testing was 2.4% and 0%, respectively. Moreover, ASD and DD/ID group received a statistically significant diagnostic yield compared to the other groups. Accordingly, a recent meta-analysis has analyzed the genetic diagnostic yields of WES and CMA in patients with DD, ID,

and/or ASD [212]. Based on this analysis, the authors proposed a diagnostic algorithm placing WES at the beginning for the evaluation of unexplained NDDs. If no genetic alteration is observed and CNV detection is not available, they recommend CMA as the second genetic test [213]. The introduction of WES as a first-tier test might provide several advantages without implying a significant increase in the turnaround time when compared to CMA. Moreover, WES offers the possibility to reanalyze negative cases or VUS findings adding new genes that can play a role in diagnosing the disease, which are continually increasing in ASD. It has been reported that WES reanalysis may increase the diagnosis yield up to ~30%, noting that this rate is not specific for ASD [214, 215].

Although the cost of WES might be a factor preventing patients from doing the test, highlighting the increased diagnostic yield and benefits of receiving a molecular diagnosis might increase parents' willingness to do the test. It is important to also discuss the fact that the broad phenotypic spectrum of ASD makes it even more challenging to reach a genetic diagnosis. In our study, the division of our sample according to clinical presentations can inform the clinicians' decisions regarding genetic testing strategy in future patients with ASD. Although a large percentage of patients remain undiagnosed, this might be explained by the multi-factorial origin of ASD, as both common and rare genetic variants contribute to autism risk. Even when a variant is identified, other multiple rare and common genetic variants contribute to the psychiatric traits in ASD patients and, thus, to the clinical and genetic heterogeneity of the disorder.

5.4 Limitations

There are some limitations in this study, including a time limitation that resulted in the inability to collect accurate family segregation analysis data, which

may have facilitated the reassessment the VUS variants identified by WES and CMA. Moreover, due to the time limitation, we could not collect medical recommendations and follow up records for our patients, which could have improved our discussion regarding the impact of the positive results in clinical management. In addition, one of the challenges was the lack of homogeneous studies for comparison given the huge differences between study designs due to the clinical complexity and genetic heterogeneity of ASD, which made it difficult to accurately compare the different findings. All these limitations could have contributed to decrease the diagnostic yield observed in our study.

5.5 Future directions

A future plan is to further study the novel candidate genes and VUS could be to work on comparing our findings with Qatar Genome Project (QGP) and Qatar Biobank (QBB) to study the frequency of our findings and possibly reclassify them.

Larger studies of a similar homogenized populations are also needed to confirm our findings. Parental and family segregation studies of identified VUS would likely improve the diagnostic yield, as well as help in shedding the light on possible reclassifications as pathogenic or benign variants, which will facilitate the process of genetic counseling for future patients with similar findings in Qatar and worldwide.

5.6 Conclusion

ASD is a complex neurodevelopmental disorder with a proven genetic basis. The genetic diagnostic yield of the disease had not been previously studied in Qatar. Our results shed light on the three most important genetic tests for pediatric patients with ASD (FXS, CMA and WES). Our findings suggest that WES could be useful in the genetic diagnosis of ASD as a first-tier test, especially nowadays when CNV

analysis from WES data is possible and methods are being increasingly optimized. WES may be followed by CMA in those unsolved cases or cases where the genomic region of a large CNV identified by WES needs to be accurately delimited. FXS testing should not be systematically used as a first-tier test; it is recommended to request FXS testing only in highly suspected cases of FXS or those with a positive family history only. These results suggest that WES would be an efficient primary diagnostic method for patients with ASD. Moreover, as patients with ASD and DD/ID tend to have the highest diagnostic yield by WES compared to other groups, our data may aid clinicians to better determine which subset of ASD patients with additional clinical features would benefit the most from WES.

Chapter 6: Appendix

Appendix A: Study approvals

مؤسسة حمد الطبية
Hamad Medical Corporation

APPROVAL LETTER
MEDICAL RESEARCH CENTER
HMC, DOHA-QATAR

Ms. Sahar Mukhtar Ibrahim Mukhtar Agouba **Quality Management Reviewer** **Administrative Service, Medical Affairs** **Hamad Medical Corporation**	**Date: 5th September 2021**
Protocol No.	MRC-01-21-667
Study Title:	Epidemiology and Genetics of Autism in the Population of Qatar: The diagnostic yield and genetic contribution of the disease
The above titled research study has been approved to be conducted in HMC and is summarized below:	
Study type:	Data Review
Data Collection Period:	01/01/2016 to 31/12/2020
Team Member List:	Dr. Nader Izz Eddin Saleem Aldewik, Dr. Noora Shabeck, Dr. Reem Jawad A A Al Sulaiman, Dr. Tawfeg I M Ben Omran, Ms. Hajer Mahmoud M A Al-Mulla, Ms. Karen El-Akouri, Ms. Sahar Mukhtar Ibrahim Mukhtar Agouba, Ms. Sara Mohd Jemmieh
Review Type:	'Exempt' under MOPH guidelines Category 3: Research involving the collection or study of existing: data, documents, records and the information is recorded by the investigator in such a manner that subjects cannot be identified, directly or through identifiers linked to the subjects.
Decision:	01/01/2016 to 31/12/2020
Hospitals/ Facilities Approved:	Hamad General Hospital (HGH)

This study must be conducted in full compliance with all the relevant sections of the Rules and Regulations for Research at HMC and the Medical Research Center should be notified immediately of any proposed changes to the study protocol that may affect the 'exempt' status of this study. Wherever amendments to the initial protocol are deemed necessary, it is the responsibility of the Principal Investigator to ensure that appropriate reviews and renewed approvals are in place before the study will be allowed to proceed.

Please note that only research documentation currently uploaded in ABHATH is to be utilized at any stage in the conduct of this study. The research team must ensure that changes and progress on the study are appropriately recorded in ABHATH, the online research system of the Medical Research Center. The PI must ensure that any link to patient identifiers is destroyed after data collection and data security is maintained.

We wish you success in this research and await the outcomes in due course.

Yours sincerely,

Prof. Michael Paul Frenneaux
Chief of Scientific, Academic and Faculty Affairs

DATE:	October 7, 2021
TO:	Houssein Khodjet Elkhil
FROM:	Qatar University Institutional Review Board (QU-IRB)
PROJECT TITLE:	1815135-1Epidemiology and Genetics of Autism in the population of Qatar: the diagnostic yield and genetic contribution of the disease
QU-IRB REFERENCE #:	QU-IRB 1609-E/21
SUBMISSION TYPE:	New Project
ACTION:	DETERMINATION OF EXEMPT STATUS
DECISION DATE:	October 7, 2021
REVIEW CATEGORY:	Exemption category # 3

Thank you for your submission of New Project materials for this project. The Qatar University Institutional Review Board (QU-IRB) has determined this project is EXEMPT FROM IRB REVIEW according to Qatar Ministry of Public Health regulations. Please note that exempted proposals do not require renewals however, any changes/modifications to the original submitted protocol should be reported to the committee to seek approval prior to continuation.

We will retain a copy of this correspondence within our records.

Documents Reviewed:

- Application Form - QU-IRB Brief Application - ASD HK KE ND MS.pdf (UPLOADED: 09/21/2021)
- Data Collection - Data Sheet final .xlsx (UPLOADED: 09/21/2021)
- Other - HMC approval .pdf (UPLOADED: 09/23/2021)
- Other - QU-IRB Check List ASD HK.pdf (UPLOADED: 09/21/2021)

If you have any questions, please contact QU-IRB at 4403 5307 or qu-irb@qu.edu.qa. Please include your project title and reference number in all correspondence with this committee.

Best wishes,

Dr. Mohamed Emara
Chairperson, QU-IRB

Institutional Review Board
(IRB)
Office Of Academic Research

This letter has been issued in accordance with all applicable regulations, and a copy is retained within Qatar University's records.

Appendix B: CMA benign, solved, and unsolved VUS

Table S1. Unsolved VUS reported from CMA

Research Number	Gender	Group	Chromosomal Region	Genomic Coordinates	Del/Dup	Size	Comment [a]	Parental results
7	Male	ASD complex	20q12.1	(39,619,764-39,915,746)	Dup	~296 kb	This copy number change results in an additional copy of *TOP1* gene.	ND
20	Female	ASD complex	Xq13.1	(69009499-69042244)	Del	~32 kb	The loss causes intragenic deletion in *EDA* gene. Loss of function mutations in EDA cause X-linked hypohidrotic ectodermal dysplasia and Tooth agenesis, selective, X-linked 1. Males have a classic clinical presentations and females have milder clinical features.	ND
31	Female	ASD complex	6q26	(162707670-162870548)	Del	~163 kb	The copy number change results in intragenic deletion of *PRK2* gene associated with autosomal recessive, juvenile (early onset) Parkinson disease 2. Heterozygous PRKN deletions, as well as intragenic duplications, particularly involving the 5' exons, are commonly observed in the general population and in control populations.	ND
68	Male	ASD complex	4q22.2	(93809184-93933335)	del	~124 kb	The deleted region contains *GRID2* gene. Recently, a patient with 276 ~kb deletion in *GRID2* gene, had spastic paraplegia, ataxia, frontotemporal dementia, and lower motor neuron disease. This deletion was a *de novo* event [216].	ND
80	Male	High functioning ASD	6q26	(161,992,369-162,437,271)	del	~445 kb	The copy number change results in intragenic deletion of *PRK2 (PRKN)* gene. The *PRK2* gene is associated with autosomal recessive juvenile Parkinson disease.	ND
112	Male	ASD non-verbal	1q43	(236,906,731-237,068,377)	dup	~161 kb	The duplicated segment contains *MTR* gene. Loss of function mutations in *MTR* gene are associated with	Maternal negative

							homocystinuria, an autosomal recessive condition.	Paternal ND*
Research Number	**Gender**	**Group**	**Chromosomal Region**	**Genomic Coordinates**	**Del/Dup**	**Size**	**Comment [a]**	**Parental results**
116	Male	ASD non-verbal	14q32	(105,080,880-107,349,540)	del	~2 Mb	The deleted segment contains BRF1 gene, mutations in this gene have been reported in patients with Cerebellofaciodental syndrome, an autosomal recessive condition.	ND
381	Male	ASD complex	17p13.2p13.1	(6071889-6538954)	dup	~467 kb	The gain causes intragenic duplication of the *KIAA0753* gene, which may disrupt this gene. Mutations in this gene have been reported to cause Orofaciodigital syndrome.	ND
410	Male	ASD complex	7q35	(145861514-145878818)	del	~17 kb	The deletion may result in disruption of *CNTNAP2* gene. Disruption of this gene has been reported in children with autism [217, 218]. However, due to the presence of additional chromosomal imbalances or potentially pathogenic mutations within many cases and/or demonstrated inheritance of the aberration from an unaffected parent, and the finding of deletions of exonic sequence of *CNTNAP2* in control populations the potential haploinsufficiency for *CNTNAP2* is not certain at this time.	ND
584	Male	ASD nonverbal	14q23.3	(67,257,994-67,471,704)	del	~214 kb	This copy number change causes partial deletion of the *GPHN* gene. Deletions of exons 3 to 5 of the GPHN gene may play a role in the risk for neurodevelopmental disorders. The biological significance of this copy number change is not well established.	ND
612	Male	ASD non-verbal	11p11.2	(44,301,575-44,305,745)	dup	~4 kb	This copy number change causes partial duplication of *ALX4* gene, which may disrupt this gene. Heterozygous deletions and mutations have been in *ALX4* gene patients with Potocki-Shaffer syndrome.	ND
634	Female	ASD complex	22q11.21	(18051498-18116459)	dup	~65 kb	The deleted segment includes *ATP6V1E1* gene. There is no disease associated with mutations in this gene. No	Not paternal

							human mutations have been reported.	Maternal ND
Research Number	**Gender**	**Group**	**Chromosomal Region**	**Genomic Coordinates**	**Del/Dup**	**Size**	**Comment [a]**	**Parental results**
650	Male	ASD nonverbal	13q21.2	(59,894,708-60,334,880)	dup	~440 kb	The copy number change may disrupt *DIAPH3* gene. Mutations in this gene are reported to cause auditory neuropathy an autosomal dominant condition. The clinical significance of this copy number change in this patient is unclear.	ND
716	Male	ASD nonverbal	3q28	(190039234-190278801)	dup	~239 kb	The duplicated segment contains *CLDN16* gene.	ND
925	Male	ASD complex	20q13.2	(50,128,386-50,931,379)	dup	~803 kb	The duplicated segment contains *SALL4* gene, associated with Duane-radial ray syndrome.	ND
847	Male	ASD complex	10q21.1	(56452462-56468863)	dup x 4	~16 kb	The gain causes intragenic duplication in *PCDH15* gene, mutations in this gene have been associated with Usher syndrome type1D/F and 1F.	ND
883	Male	ASD and ID/DD	20p13	(859815-1074907)	dup	~215 kb	The duplicated genomic segment *RSPO4* gene. Mutations in this gene cause Anonychia congenita, an autosomal recessive condition characterized by absence of fingernails and toenails with variable expression.	ND
561	Female	ASD complex	Xq11.1q11.2	(62,853,729-63,049,516)	del	~195 kb	The loss causes partial deletion of *ARHGEF9* gene, deletion, and loss of mutation in this gene have been reported in males with Epileptic encephalopathy, early infantile. Females with X abnormalities do not usually manifest because of the presence of normal X chromosome. The biological significance of this copy number change is unknown.	ND
672	Female	ASD nonverbal	Xp22.2	(10819552-10900003)	dup	~80 kb	The gain may cause intragenic duplication in *MID1* gene. Loss of function mutations in *MID1* are responsible for Opitz G/BBB syndrome, type 1 in males. Several types of loss of function mutations have been reported, including whole and partial gene deletions, splicing, nonsense, and frameshift mutations. Female carriers typically have mild hypertelorism.	ND

Research Number	Gender	Group	Chromosomal Region	Genomic Coordinates	Del/Dup	Size	Comment [a]	Parental results
			15q26.1	(91252696-91309512)	del	~57 kb	The lost causes intragenic deletion of *BLM* gene, mutations in this gene are associated with Bloom syndrome, an autosomal recessive condition.	
923	Male	ASD and ID/DD	2p22.1	(39,135,205-39,449,673)	dup	~314 kb	The duplication results in an extra copy of *SOS1* gene. Noonan syndrome phenotype caused by mutation in the *SOS1* gene that lies within the Noonan syndrome spectrum but is distinctive. Multiple patients with developmental delay and additional significant developmental and morphological phenotypes have been reported in ISCA database.	ND

Del= deletion/ dup= duplication/ a= comment according to the molecular lab/ kb= kilobase/ Mb= mega base/ ND= not done/ *the mother did the test and it came back negative however the father did not do the test

Table S2. Solved VUS by parental testing reported from CMA

Research Number	Gender	Group	Chromosomal Region	Genomic Coordinates	Del/Dup	Size	Comment [a]	Inheritance [B]
63	Male	ASD complex	10p13	(13,831,484-14,728,775)	dup	~897 kb	The copy number change causes partial duplication of *FRMD4A gene.*	Paternal
75	Male	ASD complex	3q29	(197621570-197763860)	dup	~142 kb	There are no genes in this region which are reported to be associated with autism.	Maternal
167	Female	ASD complex	3q26.32	(176,602,334-176,800,978)	dup	~198 kb	This gain causes partial duplication of the *TBL1XR1* gene, which may disrupt the gene and result in clinical phenotype.	Paternal
214	Male	ASD complex	6p13.11p12.3	(16,635,625-18,306,841)	del	~1.6 Mb	The size of the loss includes *XYLT1* gene. Deletions of this region were observed in individuals with intellectual disability, microcephaly and/or epilepsy. It is important to note that deletions of this region were also seen in some unaffected individuals in these families, but not in over 2000 normal control individuals examined. This observation may be due to incomplete penetrance or variable expressivity of the phenotype among some family members. Therefore, the significance of this deletion should be interpreted in the context of this patient's clinical presentation.	Maternal
240	Male	ASD Nonverbal	6q12	(65,625,326-66,182,206)	del	~556 kb	This loss causes partial deletion of the *RP25* gene. Mutations in this gene have been reported to cause Retinitis pigmentosa 25, an autosomal recessive condition.	Paternal
275	Male	High functioning ASD	6p21.1	(45323740-45384088)	dup	~60 kb	The copy number change may disrupt the *RUNX2* gene. Alterations in this gene have been reported in patients with Cleidocranial dysplasia an autosomal dominant condition.	Maternal
314	Male	ASD complex	2q13	(110825110-110983457	dup	~158 kb	The biological significance of this copy number change is not clear. Multiple cases with deletions and duplications in this region have been classified as likely benign variants.	Maternal

Research Number	Gender	Group	Chromosomal Region	Genomic Coordinates	Del/Dup	Size	Comment [a]	Inheritance [B]
411	Male	ASD nonverbal	22q11.21	(18,894,902-21,464,056)	dup	~2.5 Mb	The duplication segment overlaps with cat eye syndrome region and 22q11.2 duplication syndrome but smaller in size. The characteristic signs and symptoms of cat-eye syndrome, include coloboma, heart defects, kidney problems, malformations of the anus, and in some cases, delayed development.	Paternal
504	Female	ASD nonverbal	6q24.2	(144785555-145005808)	del	~220 kb	The copy number change causes intragenic deletion of *UTRN (DMDL)* gene. The UTRN gene encodes utrophin, a large skeletal muscle protein that shows similarities to dystrophin (DMD), Higley expressed in wide range of human tissues. Currently, this gene has not been associated with a clinical condition.	Maternal
511	Female	ASD complex	17q12	(34917400-34906734)	dup	~89 kb	The duplicated region includes *ZNHIT3* gene. Mutations in *ZNHIT3* have been reported in PEHO syndrome, an autosomal recessive condition. This patient has an extra copy of ZNHIT*3* gene, copy number gain of this region have been classified as benign in ISCA database.	Maternal
559	Male	ASD complex	11p15.3p15.2	(12382987-12937493)	dup x 4	~554 kb	This is 2 copy gain within *TEAD1* gene. There is possibility that the copy number change in this patient may have disrupted one or both copies of this gene depending on being in *cis* or *trans*. DECIPHER database contains two patients with deletions of similar size in this gene, one patient had cleft palate and the second patient had developmental delay.	Paternal
582	Female	ASD complex	2q32.2 q35	(191,301,612-192,053,337)	del	~751-kb	The deleted region contains *STAT1*, *NAB1* and *STAT4* genes	Maternal
615	Male	ASD complex	6p12.1	(55,932,240-56,504,572)	dup	~572 kb	The copy number change causes partial duplication of the *DST* gene. Loss of mutations in *DST* gene are associated with Neuropathy, hereditary sensory and autonomic, type VI and Epidermolysis bullosa simplex,	Paternal

							autosomal recessive 2, both conditions are autosomal recessive. This patient is reported to have autism, there is reported evidence that mutations in *DST* gene are associated with autism.	
643	Male	ASD nonverbal	14q32	(103,316,124-103,365,453)	dup	~179 kb	The change results in partial duplication of *TRAF3* gene.	Maternal
697	Male	ASD nonverbal	1q21.3	(151333185-151420636)	dup	~ 87 kb	This copy number change results in partial duplication of *POGZ* gene and may disrupt the gene. Heterozygous mutation in *POGZ* can cause White-Sutton syndrome WHSUS is a neurodevelopmental disorder characterized by delayed psychomotor development shows in infancy, and a characteristic constellation of dysmorphic facial features. A big number of patients also have autism.	Maternal
698	Male	ASD complex	6q16.3	(102175271-102530128)	del	~354 kb	The loss causes an intragenic deletion of the *GRIK2* gene. Changes in GRIK2 are associated with autosomal recessive intellectual disability.	Maternal
708	Male	ASD nonverbal	15q26.3	(99,507,726-99,568,726)	dup	NR	The gain causes intragenic duplication of the *IGF1R* gene.	Paternal
738	Female	ASD complex	7q33	(133,097,085-133,317,746)	del	~ 220 kb	The deletion results in partial deletion of the *EXOC4* gene, part of a multiprotein complex involved in vesicle trafficking at neural synapses and vesicle targeting in polarized epithelial cells.	Maternal
913	Male	ASD complex	22q11.21	(20,666,262-20,886,709)	dup	~220 kb	The copy number change results in an additional copy of *SCARF2* gene. Loss of functions mutations in this gene have been reported in patients with Van den Ende-Gupta syndrome an autosomal recessive condition.	Paternal
1007	Male	ASD and ID/DD	1p32.2	(58066376-58291809)	del	~225 kb	The loss is in size and causes intragenic deletion of *DAB1* gene.	Maternal
850	Female	ASD and ID/DD	13q31.3	(94,369,715-94,376,911)	del	~7 kb	The deletion results in partial deletion of *GPC6* gene, mutations in this gene have been reported in Omodysplasia 1, an autosomal recessive condition.	Maternal

Del= deletion/ dup= duplication/ a= comment according to the molecular lab/ b= inherited from which parent/ kb= kilobase/ Mb= mega base

Table S3. Benign variants reported from CMA

Research Number	Gender	Group	Genomic Coordinates	Chromosomal Region	Del/Dupl	Size	Comment [a]	Parental Results
19	Male	ASD complex	1314778032-131902502	2q21.1	Del	~424 kb	There is gene reported in this region, which is associated with a clinical condition.	ND
8	Male	ASD nonverbal	45786840-46119127	4p12	dup	~332 kb	There is no gene reported to be associated with a clinical condition in this region.	Inherited from healthy mother
39	Male	ASD complex	12,021,840-12,748,459	9p23	dup	~726 kb	The duplicated segment contains an OMIM gene *TYRP1*.	Inherited from healthy mother
106	Male	ASD nonverbal	49,051,052-49,208,143	2P16.3	del	~157 kb	The loss causes intragenic deletion of *FSHR* gene, reported to be associated with ovarian dysgenesis.	Imnherted from healthy mother
234	Male	ASD complex	2,581,784-2,790,441	9p24.2	dup	~208 kb	This finding has been classified as benign Copy Number Change (bCNC) in ISCA database.	ND
307	Male	ASD nonverbal	46694688-46910154	11p11.2	dup	~215 kb	Copy number changes in this region have been classified as benign / likely benign in Clingen database	ND
540	Male	ASD complex	110,814,584-111,244,046	7q31.1	del	~429 kb	This finding seems a benign Copy Number Change (bCNC)	ND
705	Male	ASD complex	17,055,384-18,207,465	12p12.3	dup	~1 Mb	There is no OMIM gene in the duplicated segment.	ND
917	Male	ASD and ID/DD	21,382,548-21,745,000	16p12.2	del	~362 kb	includes *DFNB22* gene associated with autosomal recessive deafness	Inherited from healthy mother
1030	Male	ASD complex	130913289-131151187	2q21.1	dup	~237 kb	Duplication in this region have been classified as benign in Clingen database.	Inherited from healthy father

Del=deletion/ dup= duplication/ a= comment according to the molecular lab/ kb= kilobase/ Mb= mega base/ ND= not done

Appendix C: WES non diagnostic variants and VUS

Table S4. VUS and likely benign variants reported by WES

Research number	Group	Gender	Gene	Disease	Mode of Inheritance	cDNA Level	Protein Level	Zygosity	Classification	Inheritance*
5	Non-verbal ASD	Female	*ATAD3C*	None currently described	Unknown	c.92_93insGAA	p.D31delinsEN	HT	VUS	Mother
			ATAD3C	None currently described	Unknown	c.301G>A	p.A101T	HT	VUS	Mother
			ATAD3C	None currently described	Unknown	c.631 C>T	p.R211W	HT	VUS	Father
19	ASD complex	Male	*MT-CO3* [a]	/	Maternal	m.9229 A>T	p.Y8F	HMp	VUS	Mother
25	ASD complex	Female	*CC2D2A*	Joubert syndrome and related disorders	AR	c.2387 T>G	p.M796R	HM	VUS	Mother And Father
28	ASD complex	Male	*MT-ND4* [a]	/	/	m.12134 T>C	p.S459P	HMp	VUS	Mother
33	ASD complex	Male	*SBF-1*	SBF-1 related disorder	AR	c.3317 C>T	p.P1106L	HM	VUS	Mother (HT) Father not tested
35	High functioning	Female	*SH2B3*	None currently	Unknown	c.661delAinsCGGCT	p.L224GfsX45	HT	VUS	Denovo

	ASD			described						
Research number	**Group**	**Gender**	**Gene**	**Disease**	**Mode of Inheritance**	**cDNA Level**	**Protein Level**	**Zygosity**	**Classification**	**Inheritance***
			SH2B3	None currently described	Unknown	c.772 C>T	p.Q258X	HT	VUS	Father
72	Non-verbal ASD	Male	*DEPDC5*	DEPDC5-related disorder	AD	c.4553 G>A	p.R1518H	Mosaic	VUS	Denovo
156	**ASD and ID/DD**	**Female**	***DPYD***	**DPYD related disorder**	**AR**	**c.2303 C>T**	**p.T768K**	**HT**	**VUS**	**Mother**
			DPYD	**DPYD related disorder**	**AR**	**c.95 C>T**	**p.S32L**	**HT**	**VUS**	**Father**
158	Non-verbal ASD	Male	*DYRK1A*	DYRK1A related disorder	AD	c.243 G>A	p.M81I	HT	VUS	Mother
83	ASD and ADHD	Male	*KCNQ2*	KCNQ2 related diorder	AD	c.1301 G>A	p.S43N	HT	VUS	Mother
			SMPD4	SMPD4 related disorder	Unknown	c.749 G>C	p.G250A	HM	VUS	Father And Mother
118	**ASD complex**	**Male**	***ZNF711***	**ZNF711 related disorder**	**X linked**	**c.102 A>T**	**p.Q341H**	**HMi**	**VUS**	**Mother**
125	ASD and ADHD	Male	*SOS2*	Noonan syndrome	AD	c.3379+1 G>C	IVS21+1G> C	HT	VUS	Denovo

Research number	Group	Gender	Gene	Disease	Mode of Inheritance	cDNA Level	Protein Level	Zygosity	Classification	Inheritance*
150	ASD complex	Male	*ATP6AP1*	ATP6AP1 related disorder	X linked	c.593 G>T	p.G198V	HMi	VUS	Mother
206	ASD and ID/DD	Male	*KIRREL3*	KIRREL3 related intillectual diasability	AD	c.1166 G>A	p.R389H	HT	Likely benign [a]	Mother
			SLC9A9	**SLC9A9 related disorder**	**AD**	**c.936 A>T**	**p.E312D**	**HT**	**VUS**	**Mother**
217	Non-verbal ASD	Female	*MED24*	None currently described	Unknown	c.170 A>G	p.N57S	HT	VUS	*novo*
277	High functioning ASD	Male	*NDST1*	NDST1 related disorder	AR	c.675 G>T	p.W225C	HT	VUS	*Denovo*
			ABCA2	None currently described	Unknown	c.3139 G>A	p.E104K	HT	VUS	Mother
296	**ASD complex**	**Male**	***NTNG1***	**NTNG1 related disorder**	**AD**	**c.279 T>A**	**p.D93E**	**HT**	**VUS**	**Unknown**
323	**ASD complex**	**Male**	***TSC2***	**TSC2 related disorder**	**AD**	**c.716 T>C**	**p.F239S**	**Mosaic**	**VUS**	**Denovo**
			FAM46A	None currently described	Unknown	c.269 T>G	p.F90C	HM	VUS	Unknown

			PAPPA2	None currently described	Unknown	c.4126 G>A	p.E1376K	HM	VUS	Unknown
Research number	**Group**	**Gender**	**Gene**	**Disease**	**Mode of Inheritance**	**cDNA Level**	**Protein Level**	**Zygosity**	**Classification**	**Inheritance***
396	Non-verbal ASD	Male	*NRCAM*	None currently described	Unknown	c.3805 G>A	p.D1269N	Mosaic	VUS	Denovo
438	ASD complex	Male	*SLIT3*	SLIT3 related disorder	AD	c.2210 G>A	p.R737Q	HT	VUS	Father
477	ASD and ADHD	Male	*SLC2A6*	None currently described	Unknown	c.1479 G>T	p.Q493H	HT	VUS	Denovo
			MT-ND6 [a]	/	/	/	p.M64V	HTp (2%)	VUS	Mother
516	Non-verbal ASD	Female	*ALG13*	ALG13 related disorder	X linked	c.284 C>T	p.P95L	HT	VUS	Mother
531	Non-verbal ASD	Female	*OSBP*	None currently described	Unknown	c.1854 C>G	p.Y618X	HT	VUS	Denovo
540	**ASD complex**	**Male**	***BRWD3***	**BRWD3 related disorder**	**X linked**	**c.1137 T>A**	**p.F379L**	**HMi**	**VUS**	**Mother**
387	Non-verbal ASD	Female	*NLRP12**	NLRP12 related disorder	AD/AR	c.1854 C>G	p.Y618X	HT	VUS	Mother
			*NLRP12***	NLRP12 related disorder	AD/AR	c.767 A>C	p.N256T	HT	VUS	Father

Research number	Group	Gender	Gene	Disease	Mode of Inheritance	cDNA Level	Protein Level	Zygosity	Classification	Inheritance*
570	ASD complex	Male	*ALG8*	congenital disorder of glycosylation type Ih	AR	c.719 C>T	p.S240F	HM	VUS	Father And Mother
			NARS2	combined oxidative phosphorylation deficiency 24	AR	c.506 T>A	p.F169Y	HM	VUS	Father And Mother
572	ASD complex	Female	*AMOT*	None currently described	Unknown	c.2101delA	p.T701LfsX23	HT	VUS	Denovo
575	ASD and ID/DD	Male	*CADPS2*	None currently described	Unknown	c.3069delC	p.F1024LfsX14	Mosaic	VUS	Unknown
			CADPS2	None currently described	Unknown	c.3072delT	p.F1024LfsX14	HT	VUS	Unknown
579	ASD and ADHD	Male	*MPDZ*	MPDZ related disorder	AR	c.5741 A>T	p.Q1914L	HM	VUS	Father And Mother
			MT-TQ [a]	/	/	m.4350 C>T	/	HMp	VUS	Mother
584	Non-verbal ASD	Male	*H2BFM*	None currently described	X linked	c.124 C>T	p.R42X	HMi	VUS	Mother
			MT-ND6 [a]	/	/	m.14403 T>C	p.S91G	HTp (13%)	VUS	Mother (10%)

Research number	Group	Gender	Gene	Disease	Mode of Inheritance	cDNA Level	Protein Level	Zygosity	Classification	Inheritance*
594	ASD complex	Male	*RYR3*	None currently described	Unknown	c.3991 G>T	p.A1331S	HT	VUS	Father
			RYR3	None currently described	Unknown	c.5230 C>T	p.R1744W	HT	VUS	Mother
618	ASD complex	Male	*UPF1*	None currently described	Unknown	c.1435 G>A	p.V479M	HT	VUS	Denovo
646	ASD and ID/DD	Male	*HUWE1*	HUWE1 related disorder	X linked	c.2341 C>T	p.L781M	HMi	VUS	Mother
698	ASD complex	Male	*DIPK2B*	None currently described	X linked	c.111 C>T	p.Q371X	HMi	VUS	Mother
704	Non-verbal ASD	Male	*ARHGEF 6*	mental retardation - 46	X linked	c.1089 C>G	p.Ser363Arg	HMi	VUS	Unknown
720	Non-verbal ASD	Male	*ANKHD1*	None currently described	Unknown	c.4774 G>A	p.D1592N	HT	VUS	Unknown
			DLG1	None currently described	Unknown	c.1832 A>T	p.D611V	HT	VUS	Unknown
726	Non-verbal ASD	Male	*LRP1*	None currently described	Unknown	c.2839 C>T	p.R947C	HT	VUS	Denovo
734	Non-verbal	Male	*CEP290*	CEP290	AR	c.2138 A>G	p.E713G	HT	VUS	Father

	ASD			related disorder						
			CEP290	CEP290 related disorder	AR	c.2174 A>C	p.E725A	HT	VUS	Mother
			DST	DST related disorder	AR	c.5417 A>G	p.N1806S	HT	VUS	Mother
			DST	DST related disorder	AR	c.16029 A>G	p.G5343=	HT	VUS	Father
736	ASD complex	Male	*AMER1*	AMER1 Related disorder	X linked	c.2922 G>A	p.W974X	HMi	VUS	Unknown
744	ASD and ID/DD	Male	*PDGFRB*	PDGFRB related disorder	AD	c.1193 C>T	p.A398V	HT	VUS	Mother
917	ASD and ID/DD		*WDFY3*	None currently described	AD	c.6317 C>T	p.A2106V	HT	VUS	Unknown
925	ASD complex	Male	*MITF*	MITF related disorder	AD	c.809 G>A	p.R270Q	HT	VUS	Unknown
			MT-CO3 [a]		/	m.9214 A>C	p.H3P	/	VUS	/
931	ASD and ID/DD	Male	*FLNA*	FLNA related disorder	X linked	c.4476 C>T	p.G1492=	HMi	VUS	Mother
			Deletion [a]	mitochondrial deletion syndrome	/	m.8870_15235del16366	/	6.4 deletion	VUS	Heteroplasmy Less Than 15 %
990	ASD and ID/DD	Male	*FGD1*	FGD1 related disorder	X linked	c.107 G>T	p.G36V	HMi	VUS	Mother
			LAMP2	danon disease	X linked	c.379 C>T	p.P127S	HMi	VUS	Mother

Research number	Group	Gender	Gene	Disease	Mode of Inheritance	cDNA Level	Protein Level	Zygosity	Classification	Inheritance*
1003	ASD complex	Male	*MYO6*	MYO6 related disorder	AR	c.178 G>C	p.E60Q	HM	VUS	Father And Mother
1043	ASD and ID/DD	Female	*SLC18A1*	None currently described	Unknown	c.412 C>T	p.R138W	HM	VUS	Father And Mother
1046	**Non-verbal ASD**	**Male**	***CHD8***	**CHD8 related disorder**	**AD**	**c.7325 C>A**	**p.S244Y**	**HT**	**VUS**	**Mother**
848	ASD and ID/DD	Male	*MECP2*	MECP2 related disorder	X linked	c.848 C>A	p.A283D	HMi	VUS	Mother
875	**High functioning ASD**	**Male**	***CTNND2***	**CTNND2 related disorder**	**AD**	**c.442 C>A**	**p.P148T**	**HT**	**VUS**	**Unknown**
882	Non-verbal ASD	Male	*PRRC2B*	None currently described	Unknown	c.878 A>G	p.E293G	HT	VUS	Denovo
883	ASD and ID/DD	Male	*TAF1*	TAF1 related disorder	X linked	c.4898 C>G	p.T1633S	HMi	VUS	Mother
			ATP10A	None currently described	Unknown	c.964_965delCT	p.L322VfsX26	HT	VUS	Mother
75	ASD complex	Male	*MED12*	MED12 related disorder	x linked	c.6398 C>G	p.S2133C	HMi	VUS	Mother
			LINGO1	None currently	unknown	c.1528 G>A	p.A510T	HM	VUS	Father And Mother

				described						
Research number	**Group**	**Gender**	**Gene**	**Disease**	**Mode of Inheritance**	**cDNA Level**	**Protein Level**	**Zygosity**	**Classification**	**Inheritance***
822	Non-verbal ASD	Male	*ZFHX4*	None currently described	unknown	c.5182 G>A	p.A1728T	Mosaic	VUS	*De novo*
			ZNF711	ZNF711 related disorders	X linked	c.1213 A>T	p.H405N	HMi	VUS	Mother
978	Non-verbal ASD	Male	*AHNAK*	None currently described	unknown	c.17587 T>C	p.S5863P	HM	VUS	Father And Mother
43	ASD complex	Male	*WWOX*	None currently described	AR	c.713 A>G	p.Y238C	HT	VUS	Unknown
814	ASD complex	Male	*RARS*	RARS related disorder	AR	c.1347-13 T>A	INV11-13 T>A	HT	VUS	Mother
			RARS	RARS related disorder	AR	c.161 G>A	p.R54Q	HT	VUS	Father
			TBL1X	None currently described	X linked	c.266 A>G	p.N89S	HT	VUS	Mother
825	ASD and ID/DD	Male	*KCNJ6*	kappen lubinsky syndrome	AD	c.974dupA	p.Y325X	HM	VUS	Unknown
850	ASD and ID/DD	Female	*EIF3L*	None currently described	unknown	c.416 A>C	p.H139P	HM	VUS	Father And Mother

940	ASD complex	Male	*PUM2*	None currently described	unknown	c.1165 G>A	p.G389R	HT	VUS	Denovo

*Inherited from which parent/ AR= autosomal recessive/ AD=autosomal dominant/ HM= homozygous/ HT= heterozygous/ HMi= hemizygous/ HTp= heteroplasmic/ HMp= homoplasmic/ a= previously reported as VUS then was reclassified into benign by the lab/ **Variant in bold are solved VUS and suspected diagnostic variants by clinical assessment**

Table S5. WES pathogenic non-diagnostic variants

Research number	Gender	Group	Gene	Disease	Mode of Inheritance	cDNA Level	Protein Level	Zygosity	Classification	Inheritance
158	Male	ASD nonverbal	*BRCA2* [c]	Hereditary breast and ovarian cancer	AD	c.6754delT	p.S2252LfsX28	HT	Pathogenic	Father
Hereditary breast and ovarian cancer										
306	Male	ASD complex	*MT-TS1* [a]	/	/	/	m.7471dupC	HTp (2%)	Pathogenic	/
Very low heteroplasmic level										
350	Male	ASD complex	*SLC19A2*	SLC19A2 related disorder	AR	c.1063 A>C	p.K355Q	HM	Likely pathogenic	Unknown
Thiamine-responsive megaloblastic anemia syndrome (TRMA) is an autosomal recessive disorde typically presents within infancy and adolescence										
601	Male	ASD nonverbal	*DSG2* [c]	Arrhythamogenic right venticular cardiomyopathy	AD	c.82-2A>G	IVS2-2A>G	HT	Pathogenic	Father
Arrhythamogenic right venticular cardiomyopathy										
773	Female	ASD nonverbal	*TYR*	TYR related disorder	AR	c.1037-7 T>A	IVS2-7 T>A	HT	Pathogenic	Father
			TYR	TYR related disorder	AR	c.1205 G>A	p.R402Q	HT	Risk allele [b]	Mother
Oculocutaneous albinism type 1 (OCA1) is associated with reduced production of melanin in the skin, hair and eyes. OCA1 is an autosomal recessive disorder, the combined presence of pathogenic variant and risk allele in this individual may explain the presence of hypopigmented skin patches										
917	Male	ASD and ID/DD	*BCKDHA*	BCKDHA related disorder	AR	c.347 A>G	p.D116G	HT	Likely pathogenic	Unknown
Homozygous or compound heterozygous pathogenic variant cause Maple syrup urine disease (MSUD), this patient is carrier										
947	Male	ASD complex	*LDLR* [c]	familial hypercholesterol	AD	c.1291 G>A	p.A431T	HT	Pathogenic	Mother

				emia						
				Familial hypercholesterolemia						
Research number	**Gender**	**Group**	**Gene**	**Disease**	**Mode of Inheritance**	**cDNA Level**	**Protein Level**	**Zygosity**	**Classification**	**Inheritance**
1003	Male	ASD complex	*COL9A1*	COL9A1 related disorder	AD/AR	c.1411 C>T	p.R471X	HM	Pathogenic	Father and mother
				Autosomal dominant multiple epiphyseal dysplasia						
1009	Female	ASD nonverbal	*PLCB1*	PLCB1 related disorder	AR	c.1285 C>T	p.R429X	HT	Likely pathogenic	Denovo
				Early onset epileptic encephalopathy. This patient is carrier						
825	Male	ASD and ID/DD	*HBB*	HBB related disorder	AD/AR	c.118 C>T	p.Q40X	HT	Pathogenic	Unknown
Variants in HBB gene Sickle cell, beta thalassemia or hemoglobin C disease, this patient is carrier however might present with mild microcytic anemia										
			HEXA	tay sachs disease	AR	c.2 T>C	p.M1?	HT	Pathogenic	Unknown
Tay sachs disease in case of homozygous or compound heterozygous, this patient is carrier										
940	Male	ASD complex	*PRPT2*	PRPT2 related disorder	AD	c.649dupC	p.R217PfsX8	HT	Pathogenic	Father
Benign familial infantile seizure, **Paroxysmal kinesigenic dyskinesia with infantile convulsions**										

a= mitochondrial variant/ b= risk allele is an allele that confers the developing of a disease, in this patient the risk allele is associated with OCA1/ c= ACMG secondary findings/ AR= autosomal recessive/ AD=autosomal dominant/ HM= homozygous/ HT= heterozygous/ HTp= heteroplasmic

Chapter 7: References

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